PROTEOMIC STUDY OF GONADOTROPIN-RELEASING HORMONE TREATED GONADOTROPE CELL LINE

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Summary

Gonadotropin-releasing hormone (GnRH) is the first key hormone of reproduction. The binding of GnRH to its cognate type I receptor initiates diverse signaling cascades. Among them, the GnRH-induced MAPK cascades are the most studied and characterized, which are proved to facilitate the transcriptional regulation of gonadotropins. Besides MAPK signaling cascades, however, recent studies indicate that GnRH-induced gonadotropin production may also be regulated through other signaling pathways. In addition, the regulation of gonadotropins is implicated to not only occur at the transcriptional level but also at the post-transcriptional, translational and posttranslational levels. Moreover, the gonadotropins are found to be differentially regulated by GnRH pulse frequency. However, the mechanisms by which gonadotropins decipher the GnRH pulse frequency are yet to be clear. We performed cICAT proteomic profiling to compare the whole cell lysate from mock-treated and GnRH-treated LβT2 cells, and identified a number of promising factors involved in mediating GnRH response through Wnt signaling. Furthermore we did different GnRH frequency treatments in LβT2 cells, and performed iTRAQ-based proteomic experiment to compare nuclear cell lysate from mock-treated and GnRH-treated LβT2 cells. Gene ontology study identified the top-ranked biological processes, in which the identified proteins are involved. Among these, certain numbers of regulated proteins are found to be involved in mRNA processing, translation and chromosome organization, which are closely correlated to posttranscriptional, translational and posttranslational regulations, suggesting the novel regulatory roles of GnRH other than transcriptional regulation. Besides, the functions of

these regulated proteins as well as the percentile of the regulated proteins fell in each function category were found to be differentially distributed in different GnRH frequency treatment group, suggesting that the functions of these proteins are correlated with underlying GnRH decoding mechanism. Furthermore, ACTN4 was identified as a negative regulator of FSHβ subunit-gene transcription and its C-terminal Cam-like domain was required for the regulation. Our findings suggested that GnRH-induced ACTN4 nuclear translocation may facilitate the transcriptional activation of FSHβ subunit-gene, possibly through mediating the crosstalk of Ca^{2+} signaling with the MAPK pathways. This study provided valuable clues and suggestions for the future researchers to explore the underlying GnRH-induced regulatory mechanism, as well as potential drug targets for the therapy of reproductive aberrancy and deficiency related diseases.

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List of Abbreviations

2-D LC	2-Dimensional liquid chromatography
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
AA	arachidonic acid
aa-RSs	aminoacyl-tRNA synthetases
aa-tRNAs	aminoacyl-tRNAs
ACN	acetonitrile
Ap-1	Activator protein 1
AS	alternative splicing
CaM	Calmodulin
CaMKs	CaM-dependent kinases
cAMP	intracellular cyclic-AMP
CaN	calcineurine
CG	chronic gonadotropins
CHCA	α-cyano-4-hydroxycinnamic acid
cICAT	cleavable ICAT
CKI	casein kinase I
C.I.	confidence interval
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1
DAG	diacylglycerol
DAPI	4’6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DIGE	different gel electrophoresis
DMEM	Dulbecco’s modified Eagle’s medium
EF	error factor
Egr-1	Early growth factor 1
eIFs	eukaryotic initiation factors
ERK	extracellular signal regulated kinase
ESI	Electrospray Ionization
FDR	False Positive Rate
FSH	follicle stimulating hormone
FZD	Frizzled
GnRH	Gonadotropin releasing hormone
GnRHa	GnRH agonist
GPCRs	G-protein-coupled receptors
GSK-3β	glycogen synthase kinase-3β

HATs	histone acetyl transferases
HDAC	histone deacetylase
hnRNPs	heterogeneous nuclear ribonucleoproteins
HPG	hypothalamic-pituitary-gonadal
ICAT	Isotope-coded affinity tags
IP3	inositol 1,4,5-trisphosphate
IPA	Ingenuity pathway analysis
IPI	International Protein Index
iTRAQ	isobaric tag for relative and absolute quantitation
JNK	c-jun NH2-terminal kinase
LC	Liquid chromatography
LEF	lymphoid enhancer factor
LH	luteinizing hormone
LHR	LH receptor
m/z	mass-to-charge
MALDI	Matrix Assisted Laser Desorption Ionization
MAPK	mitogen-activated proteins kinase
MEF2	myocyte enhancer factor-2
MEM	Modified Eagle Medium
MKKs	MAP kinase kinases
MMTS	methyl methanethiosulfonate
MS	mass spectrometry
NFAT	nuclear factor of activated T
NFY	nuclear transcription factor-Y
NuRD	Nucleosome Remodeling and Deacetylase
PCOS	polycystic ovarian syndrome
pcv	packed cell volume
PIP2	phosphatidylinositol 4,5-bisphosphate
Pitx-1	Pituitary homeobox 1
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
pnv	packed nuclear volume
PTMs	post-translational modifications
Rbm	RNA binding motif
RGS	Regulators of G-protein signaling
RP	reverse-phase
RRMs	RNA recognition motifs
RT	room temperature
S.D.	standard deviation
SAPKs	stress activated proteins kinases

SCX	strong-cation exchange
Sf-1	Steroidogenic factor 1
shRNA	short hairpin RNA
snRNAs	small nuclear RNAs
Sp-1	Specificity protein 1
SR	serine/arginine-rich
STRING	Search Tool for the Retrieval of Interacting Genes
TCEP	triscarboxyethylphosphine
TCF	T-cell factor
TEAB	triethylammonium bicarbonate
TFA	trifluoroacetic acid
TRPC3	receptor potential channel 3
TSH	thyroid stimulating hormone
VSCC	voltage-sensitive calcium channel
αGSU	α glycoprotein hormone subunit

List of Publications

1. • Han Yu, Zhengjun Li, Dipanjana Ghosh, Teck Kwang Lim, Yuehui He, Qingsong Lin. α-Actinin4 nuclear translocation mediates gonadotropin-releasing hormone stimulation of follicle-stimulating hormone β-subunit gene transcription in LβT2 cells. *FEBS Letters* Volume 586, Issue 10 , Pages 1466-1471, 21 May 2012

Chapter 1. Literature review

1.1 Gonadotropins and Gonadotropin-Releasing Hormone

1.1.1 Physiology of the gonadotropins

Reproduction plays critically important roles in sustentions and evolution of any animal's life. In all vertebrates, reproduction is controlled by the brain through the highly conserved hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.1). Gonadotropin releasing hormone (GnRH) neurons, which are located in the hypothalamic-preoptic area of the brain, ultimately control reproduction by incorporating internal information such as nutritional and hormonal state with those social and environmental signals. This integration results in the production and release of GnRH to the pituitary gland. GnRH is highly conserved across all vertebrates and is the pivotal regulator of gonadotopins. It was recently also found in several invertebrates, emphasizing its evolutionary importance (Tsai 2006).

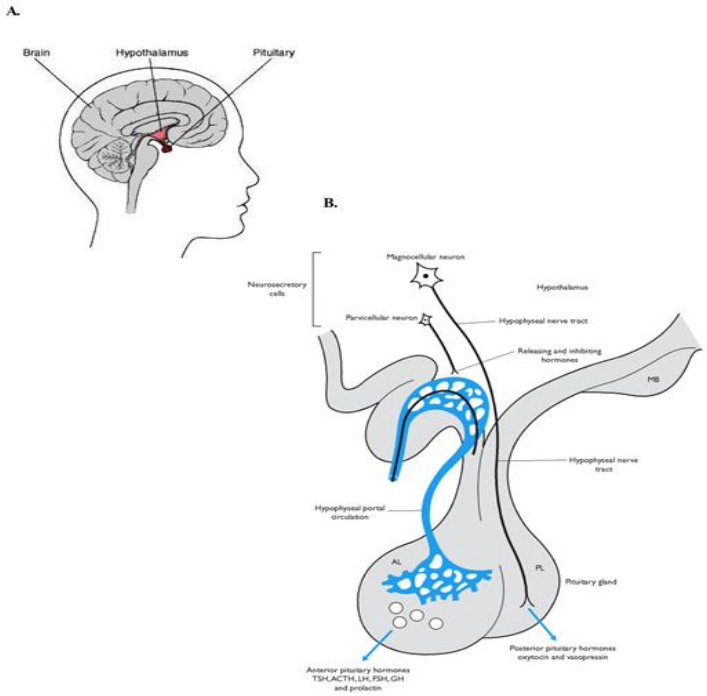


Figure 1.1 Schematic representation of the hypothalamic-pituitary-gonadal axis.

(A) The pituitary gland is a small, bean-sized organ, which is beneath the brain in a well-protected position. (B) GnRH is secreted from the hypothalamus. After binding with the GnRH receptors on the surface of the anterior pituitary gonadotrope, it acts on the gonads to stimulate the synthesis and secretion of LH and FSH (Brown and McNeilly 1999).

1.1.2 Subunits of gonadotropins

The gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) belong to the glycoprotein hormone family, which also includes thyroid stimulating hormone (TSH) and chronic gonadotropins (CG) (Pierce and Parsons 1981). LH and FSH are heterodimeric hormones sharing a common α-subunit, while owning a hormone specific β-subunit (Figure 1.2). The β-subunit specifies the biological activity and physiological particularity of each gonadotropin (Gharib, Wierman et al. 1990). The common α and specific β-subunits are localized on different chromosomes. The coordinated transcription and non-covalent assembly of these two subunits control the synthesis of biologically active LH and FSH.

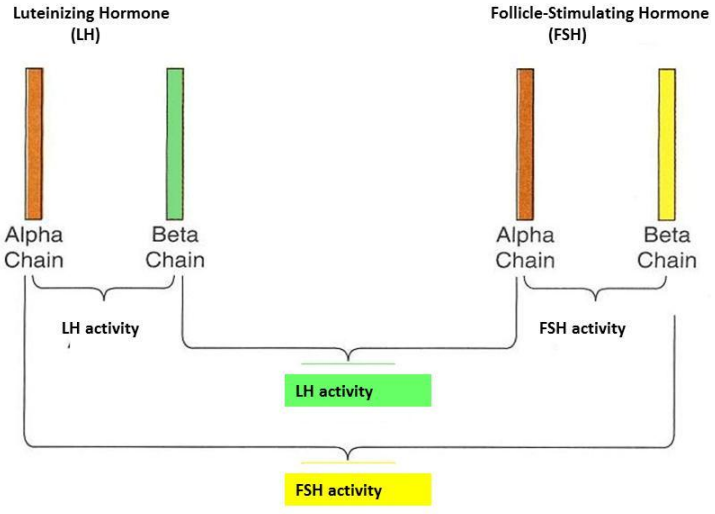


Figure 1.2 Schematic demonstration of subunits of gonadotropins.

Each of LH and FSH has a common α-subunit (orange), and a hormone-specific β-subunit (Gharib, Wierman et al. 1990).

1.1.3 Biological functions of the gonadotropins

LH and FSH initiate sexual maturation and maintain cyclical reproductive function upon binding to their specific receptors in the gonad (Figure 1.3). In the testis, LH acts on the Leydig cells to stimulate synthesis and secretion of steroid hormones, especially the production of androgens (Dufau 1988), while FSH targets the Sertoli cells to stimulate production of sperms (Tapanainen, Aittomaki et al. 1997). In the ovary, FSH stimulates maturation of ovarian follicles and conversion of androgens to estrogens (Aittomäki, Herva et al. 1996; Kumar, Wang et al. 1997), while LH promotes maturation of follicular cells. After the initial LH receptor (LHR) expression in the small follicles, LH enhances the subsequent stages of follicular development in granulosa and luteal cells (Richards and Hedin 1988). The preovulatory LH surge triggers ovulation of mature follicles by promoting the rupture of the follicle and the release of the ovum. In addition, LH also leads to the synthesis and subsequent release of progesterone by the *corpus luteum* (Norris 1997).

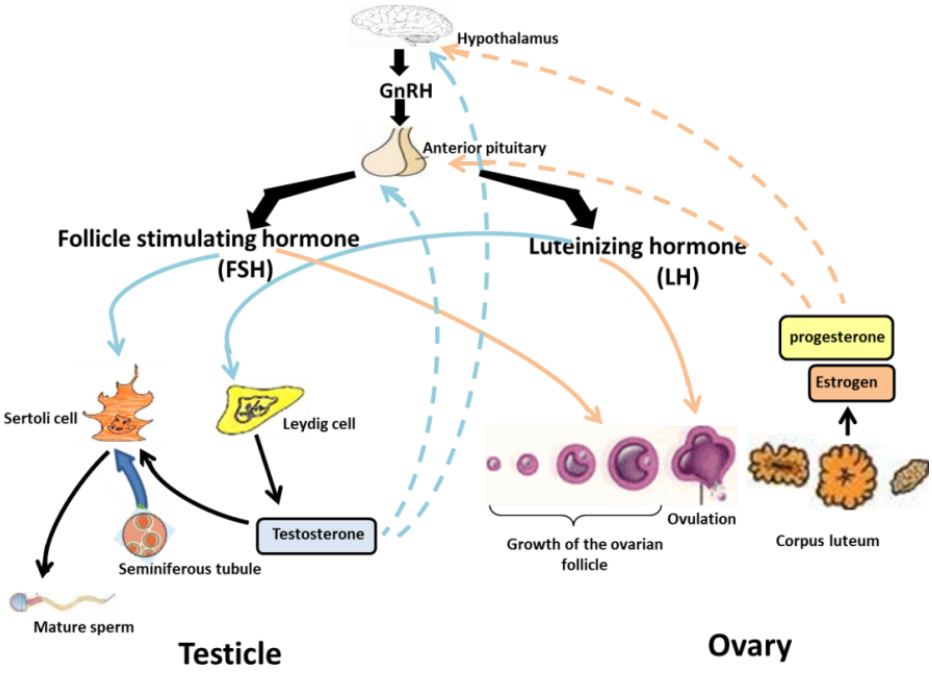


Figure 1.3 Schematic demonstration of biological functions of gonadotropins.

LH and FSH produce steroid hormones: testosterone, estrogen and progesterone in gonads, which in turn positively or negatively regulate the synthesis of gonadotropins (Brown and McNeilly 1999).

1.1.4 Therapeutic applications of GnRH

GnRH agonist (GnRHa) analogs, peptides mimic the function of GnRH, have been used to treat steroid-dependent diseases (Schultze-Mosgau, Griesinger et al. 2005) and proved to be a safe and efficient class of drugs. The existing treatments using GnRHa medication include precocious puberty, endometriosis, fibroids, breast cancer, and prostate cancer (Beyer, Amari et al. 2011). Typical side effects caused by estrogen withdrawal are acceptable for cancer treatment, and can be effectively reliefed through "add back" therapy, which utilizes combination of low dose estrogen and progesterone (Moghissi, Schlaff et al. 1998; Schlaff 1999).

1.2 Molecular regulation of gonadotropin synthesis and secretion

The synthesis and secretion of gonadotropins LH and FSH are regulated by a series of factors along the hypothalamus-pituitary-gonad axis, including gonadotropin-releasing hormone (GnRH), steroid hormones (estrogen, androgen and progesterone) and gonadal peptides (activin and inhibin) (Landefeld, Kepa et al. 1983; Ling, Ying et al. 1986; Papavasiliou, Zmeili et al. 1986; Gharib, Wierman et al. 1990). GnRH is the main regulator of gonadotropins. It is secreted from hypothalamus and delivered to the gonadotrope in pulses of different frequency and amplitude (Haisenleder, Katt et al. 1988; Dalkin, Haisenleder et al. 1989; Haisenleder, Dalkin et al. 1991; Kirk, Dalkin et al. 1994). This pulsatile secretion of GnRH results in the differential regulation of the expression of LHβ and FSHβ. Gonadal steroids and peptides act either on the hypothalamus to alter GnRH pulsatility or on the pituitary to regulate LH and FSH synthesis and secretion (Landefeld, Kepa et al. 1984; Dalkin, Paul et al. 1992; Roy, Angelini et al. 1999). Current knowledge about GnRH-induced regulation of gonadotropin genes are mainly in two aspects: one is the factors involved in the regulation of basal transcriptional machinery, which include *cis*-elements and transcription factors mediating the GnRH signaling to the LHβ and FSHβ promoters, the other is GnRH-induced signaling pathways.

1.2.1 Basal level regulation of gonadotropins

1.2.1.1 Transcriptional machinery on the LHβ promoter

The mammalian LHβ promoter contains two regions: the proximal and distal regions. The proximal 140 bp region is highly conserved across all species. It contains Pituitary homeobox 1 (Pitx-1), Steroidogenic factor 1 (Sf-1) and Early growth factor 1 (Egr-1) binding sites, which act functionally and synergistically to mediate the basal and GnRH-

stimulated LHβ promoter activity, by recruiting other coactivators to the promoter (Halvorson, Ito et al. 1998; Dorn, Ou et al. 1999; Tremblay and Drouin 1999; Quirk, Lozada et al. 2001). Sf-1 is an orphan nuclear receptor found in the gonadotropes of the anterior pituitary and also in other non-pituitary steroidogenic tissues including the adrenal glands, gonads, and placenta and the ventromedial nucleus of the hypothalamus (Ikeda, Lala et al. 1993; Luo, Ikeda et al. 1994; Ikeda, Luo et al. 1995). Sf-1 knockout mice show significantly decreased expression of both α glycoprotein hormone subunit （αGSU）and LHβ (Shinoda, Lei et al. 1995). Pitx1 is expressed in several anterior pituitary cell lineages and transactivates most of the anterior pituitary cell-specific genes (Tremblay, Lanctôt et al. 1998). Egr-1 is a zinc finger transcription factor that binds to a GC-rich sequence in DNA to activate gene transcription. Targeted disruption of Egr-1 resulted in the selective loss of LH synthesis and secretion (Lee, Sadovsky et al. 1996).

In contrast to the proximal promoter, the sequence of the distal region of the mammalian LHβ promoter is not well conserved across the species. Specificity protein 1 (Sp-1) and CArG binding sites have been identified in the distal domain of the rat LHβ promoter sequence, while in the bovine LHβ promoter, the corresponding region is a nuclear transcription factor-Y (NFY) binding site instead (Kaiser, Sabbagh et al. 1998; Kaiser, Sabbagh et al. 1998; Weck, Anderson et al. 2000). The Sp-1 region in the rat LHβ promoter plays an important role in conferring GnRH responsiveness, while the role for NFY in mediating GnRH effect has not been established (Kaiser, Halvorson et al. 2000; Keri, Bachmann et al. 2000; Jorgensen, Quirk et al. 2004). The arrangement of above mentioned transcriptional factors is demonstrated in Figure 1.4(a).

1.2.1.2 Transcriptional machinery on the FSHβ promoter

The mechanisms of the basal-level regulation of the FSHβ subunit gene are less well understood compared to LHβ gene. The Activator protein 1 (AP-1) factors, Jun and Fos, are early response genes, which are up-regulated rapidly by GnRH treatment, and have been implicated in the GnRH-induced regulation of FSHβ gene, reviewed by (Melamed, Kadir et al. 2006). Several potential binding sites for the Ap-1 factors have been identified on the mammalian FSHβ promoter, and some of which are shown to be functional. However, differences were seen in promoters from various species and/or under different experimental conditions (Miller, Shafiee-Kermani et al. 2002; Chong, Wang et al. 2004; Wang, Fortin et al. 2008). In the murine FSHβ promoter, a half Ap-1 binding site is found to partially overlap with the nuclear factor Y (NFY)-binding site. But this phenomenon is not present in ovine and bovine FSHβ promoter (Coss, Jacobs et al. 2004). Another early response gene, Nur77, is also found on murine FSHβ promoter, and it plays an important role in regulating GnRH-induced de-repression of the murine FSHβ gene by recruiting other regulatory factors (Lim, Luo et al. 2007). The arrangement of above mentioned transcriptional factors is demonstrated in Figure 1.4(b).

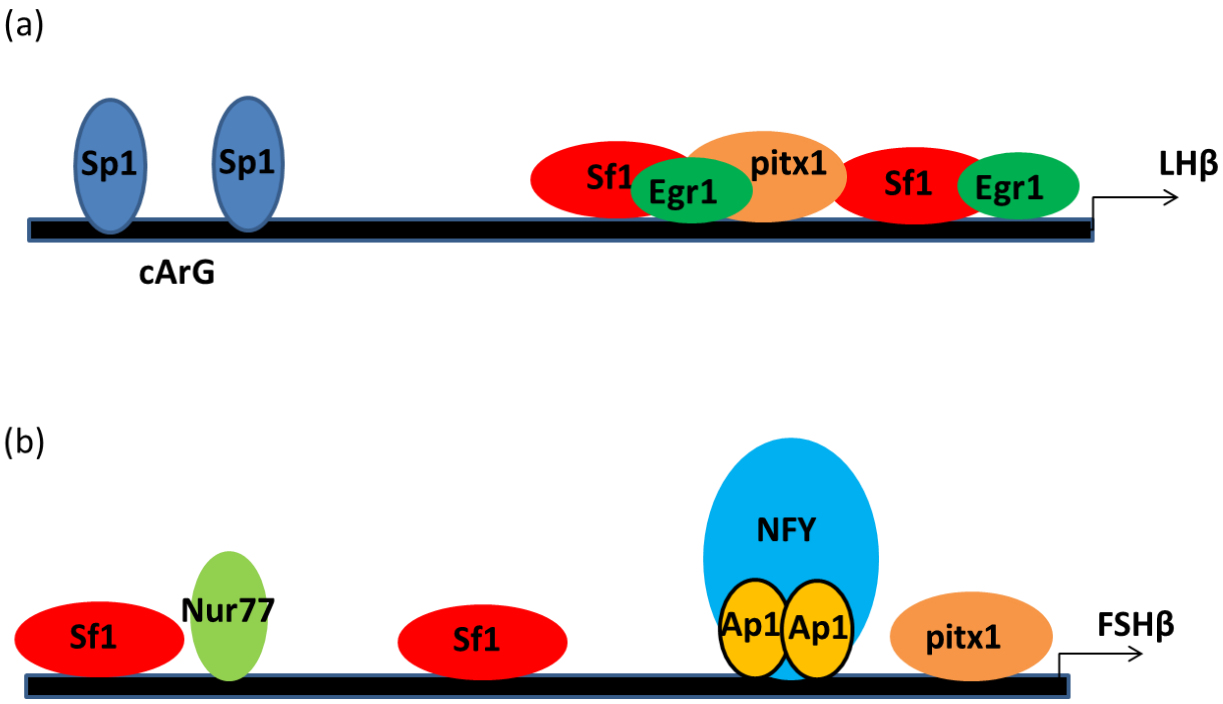


Figure 1.4 Schematic representation of transcription factors on LHβ gene and FSHβ gene promoters.

(a) On the LHβ gene proximal promoter each of Sp1, Egr1 and Sf1 has two binding sites, and pitx1 has one binding site. (b) On the FSHβ gene promoter, two Sf1 binding sites and an overlapped half Ap1 site with NFY site, a Nur77 binding site, and a pitx1 site are presented (Melamed, Kadir et al. 2006).

1.2.2 GnRH-induced regulation of gonadotropins

The GnRH signaling is initiated by the interaction of GnRH with its cognate type I receptor, GnRHR, in pituitary gonadotropes and results in the activation of Gq/G_{11}, phospholipase Cβ (PLCβI), PLA2, and PLD (Hsieh and Martin 1992). GnRHR activation induces activation of phospholipases C (PLC) and an increase in intracellular cyclic-AMP (cAMP) levels (Bourne 1988). The elevated cAMP levels activate the downstream protein kinase A (PKA) (Yoshida, Hattori et al. 1975), while PLC accelerates the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2). The cleavage consequently stimulates the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Andrews and Conn 1986). IP3 induces the calcium release from the intracellular stores, and also extracellular calcium influx through L-type voltage-sensitive calcium channel (VSCC) (Naor 1990). DAG activates protein kinase C (PKC), which activates the downstream mitogen-activated proteins kinase (MAPK) pathways, including extracellular signal regulated kinase (ERK), c-jun NH_2-terminal kinase (JNK) and p38 (Mitchell, Sim et al. 1994; Roberson, Zhang et al. 1999; Liu, Austin et al. 2002). Ca^{2+}, together with various PKC isoforms, MAPKs and arachidonic acid (AA) metabolites serve as key nodes in the GnRH-stimulated signaling network that enable the gonadotropes to decode GnRH pulse frequencies and translate them into differential gonadotropin synthesis and release (Figure 1.5)(Naor 2009).

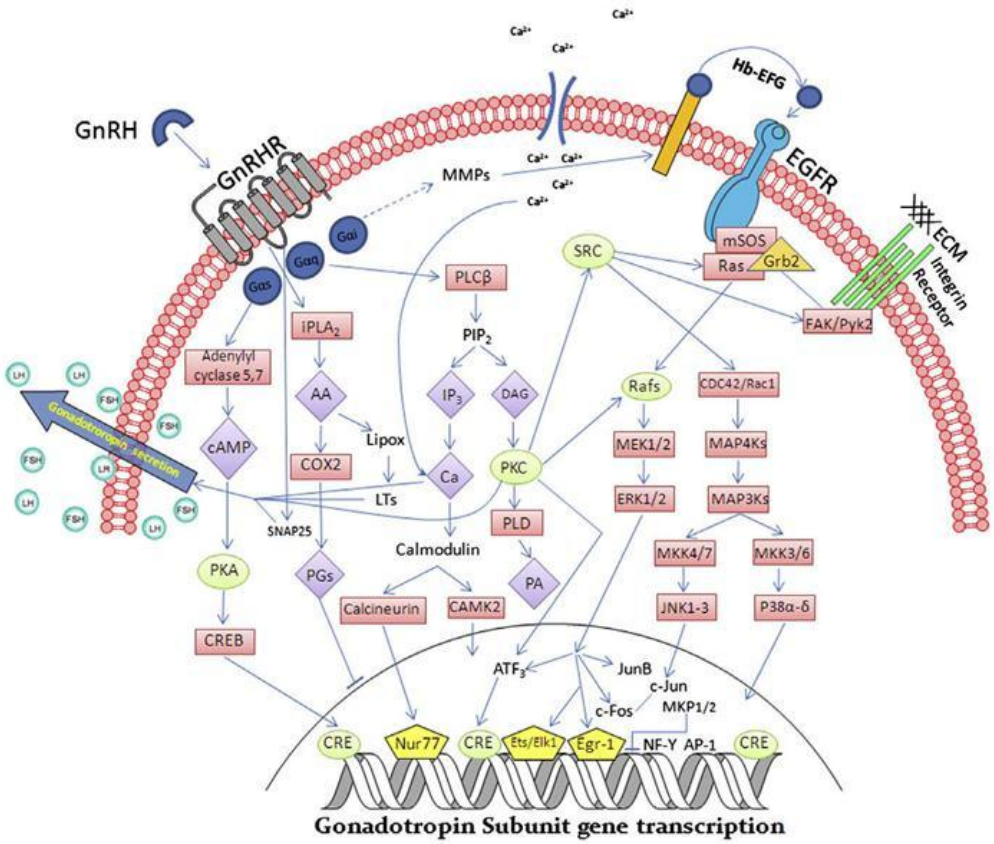


Figure 1.5 Schematic representation of GnRH signaling in gonadotrope cells

GnRH acts on the GnRHR and generates a set of selective interaction networks, which subsequently regulate the gonadotropin synthesis and secretion. It has been proposed that PKC, ERK, JNK, p38, Ca^{2+}, c-SRC play as major key nodes in the interaction network maps. This drawing is adapted from Naor, Z (Naor 2009) with permission.

1.2.2.1 MAPK signaling pathways

MAPK signaling pathways have pivotal roles including fate determination, differentiation, proliferation, survival, migration, growth and apoptosis (Pearson, Robinson et al. 2001). MAPK cascades are composed of up to six grades of protein kinases, which sequentially activate one another by phosphorylation (Seger and Krebs 1995) (Figure 1.6). Four major MAPK cascades in mammals are ERK 1-2 (p42 and p44), JNK 1-3, p38α-δ, and ERK5. They are also the most well studied signaling cascades induced by GnRH in gonadotrope cells. Previous studies have demonstrated that GnRH stimulates ERK, JNK, p38 and ERK5 in the gonadotrope cell lines (Naor, Benard et al. 2000; Benard, Naor et al. 2001; Bonfil, Chuderland et al. 2004).

The hallmark of the MAPK family is their ability to translocate into the nucleus to activate transcription factors (Seger and Krebs 1995). ERK was found to translocate into nucleus by GnRH induction The nucleus translocation of ERK results in the phosphorylation and activation of various transcription factors such as c-Fos, c-Jun, ELK-1, Ets, STAT, CREB, histone H3, Egr1, and others (Liu, Austin et al. 2002; Bonfil, Chuderland et al. 2004; Caunt, Finch et al. 2006). JNK and p38 both belong to stress activated proteins kinases (SAPKs), which are activated by stress stimuli, various GPCRs, inflammatory cytokines and growth factors. JNK cascade is initially activated by Rac1/ Cdc42, the activated signals are then transmitted through several MAP kinase kinases (MKKs) and finally activates several transcription factors such as c-Jun, ATF-2, NF-ATc1, HSF-1, STAT3, and others. The p38 cascade is also initiated by Rac1/ Cdc42, and finally activates transcription factors such as ATF-1, ATF-2, MEF2A, SAP-1, Elk-1,

NFκB, Ets-1, CHOP, p53 and others (Seger and Krebs 1995; Pearson, Robinson et al. 2001).

The roles of PKC, Ca^{2+}, and MAPK in regulating ganadotropin subunit gene expression are controversial. Murata's group claimed that JNK is involved in GnRH regulation of LHβ but not PKC and ERK (Yokoi, Ohmichi et al. 2000). However, Naor's group reported that PKC, ERK and JNK, but not Ca^{2+}, regulate LHβ gene expression (Harris, Bonfil et al. 2002). Similar controversial results were also reported for FSHβ gene (Bonfil, Chuderland et al. 2004). These conflicting results were likely due to different cell type, promoters, species, and cell culture conditions (Naor 2009).

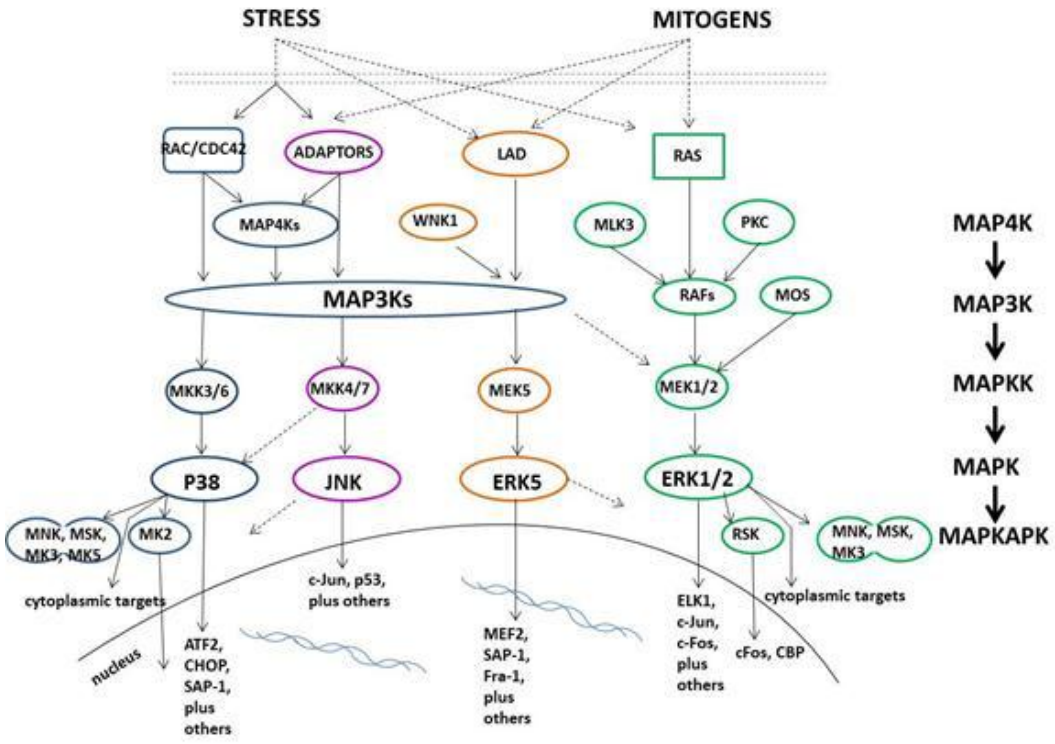


Figure 1.6 Schematic representation of the MAPK cascades.

MAPK cascades consist of up to six tiers of protein kinases that sequentially activate one another by phosphorylation. LAD is an adaptor protein that activates ERK5; WNK is a MAP4K in ERK5 pathway; MOS is an oncogene that activates ERK; RSK serves as a MAPKAPK in the ERK1/2 pathway; MSK is a MAPKAPK that can be activated by both ERK and p38. This drawing is adapted from Naor, Z (Naor 2009) with permission.

1.2.2.2 Calcium signaling pathway

Calcium ions (Ca^{2+}) represent a ubiquitous intracellular second messenger with tremendous versatility (Berridge, Lipp et al. 2000). The versatility of Ca^{2+} is based on its binding kinetics, varying amplitude, spatial-temporal distribution and multiple cross talks with other signaling cascades. The most studied Ca^{2+} downstream pathway is MAPK pathway, which was induced by elevation of cytosolic Ca^{2+} levels caused by both calcium influx and mobilization from intracellular stores. Accumulating studies have found that Ca^{2+} is required for expression of gonadotropin genes (Naor 2009). Moreover, the intermittent changes in Ca^{2+} has been suggested to be involved in decoding the GnRH frequency (Haisenleder, Yasin et al. 1997). Besides, Ca^{2+} was also found to activate alternative downstream pathway, independent of MAPK. Calmodulin (CaM) is the most notable effecter, through interaction with various kinases, phosphatases and ion channels, mediating diverse Ca^{2+} downstream pathways (Chin and Means 2000; Means 2008; Swulius and Waxham 2008). In gonadotrope cells, so far there are two well characterized CaM downstream pathways; one is the CaM-dependent kinases (CaMKs) pathway, and the other is calcineurin pathway. Both of them have been found to mediating GnRH-induced signal to gonadotropins (Melamed, Savulescu et al. 2012) (Figure 1.7).

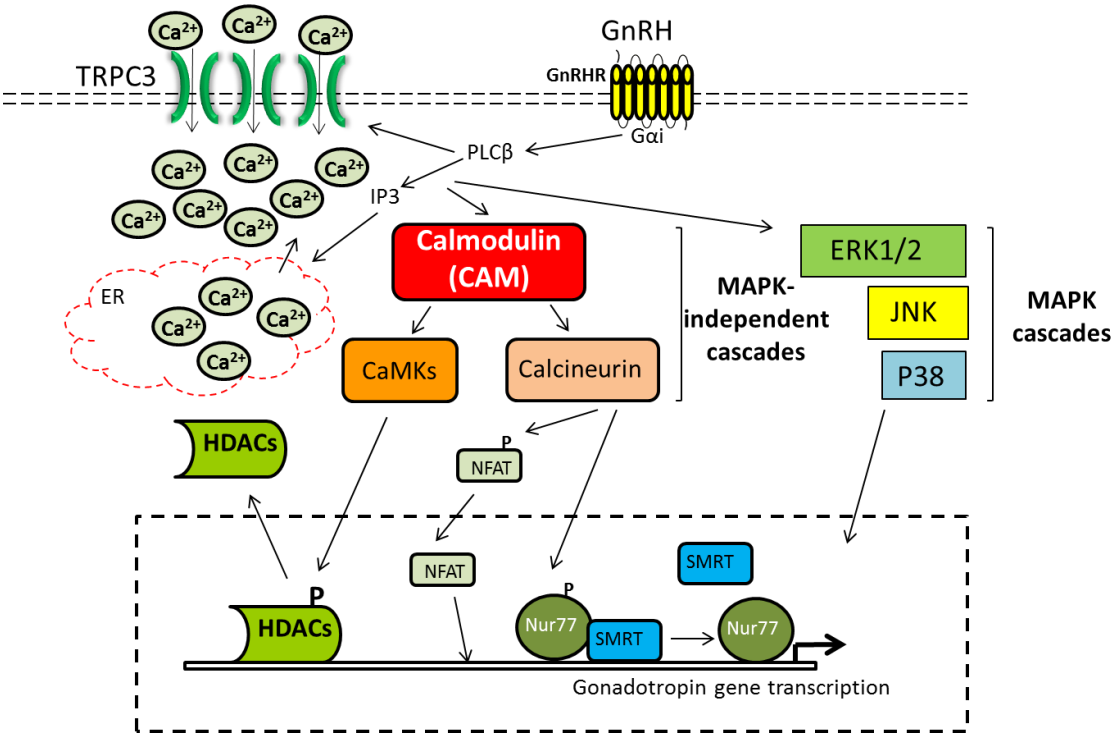


Figure 1.7 Schematic representation of Ca^{2+} signaling in gonadotrope cells.

GnRH binding to GnRHR induces calcium influx (as well as mobilization: not shown) which activates CaM. The activation of CaM consequently stimulates the downstream effectors CaMKs and calcineurin, and results in the gonadotropin expression. This drawing is based on the model proposed by Melamed *et al.*(Melamed, Savulescu et al. 2012).

1.2.2.3 Wnt signaling pathway

The WNT signaling controls numerous biological processes throughout development and adult life of all animals. Aberrant Wnt signaling underlies a wide range of pathologies in humans (Clevers and Nusse 2012). The WNT pathways are presented as two forms: the canonical Wnt/ β-catenin pathway and non-canonical Wnt pathways. The canonical Wnt/ β-catenin cascade is the best characterized Wnt signaling pathway, and β-catenin is the key effecter of this cascade. Without the stimulation of Wnt ligand on the Frizzled (FZD) family of receptors, β-catenin is captured by a destruction complex, which is composed of adenomatous polyposis coli and axin, bound by casein kinase I (CKI) and glycogen synthase kinase-3β (GSK-3β). GSK-3β phosphorylates β-catenin and targets it for ubiquitination and subsequent degradation via the proteasomal degradation pathway, and the cellular β-catenin amount is thus kept at a low level (Huelsken and Behrens 2002; Moon, Kohn et al. 2004; Nelson and Nusse 2004). The non-canonical Wnt cascades are less well characterized than their canonical counterpart. These pathways, including Wnt/ Ca^{2+} pathway and Wnt/c-JNK pathway, are also activated by Wnt ligands binding to FZD receptors, and are implicated to regulate the biological processes such as cell polarity, cytoskeleton reorganization and cell movement. A number of signaling mediators and transcription factors such as PKC, Ca^{2+}/ CaM, calcineurine (CaN), Ca^{2+}/ CaM-dependent Kinase II, nuclear factor of activated T cell (NFAT), dishevelled, RhoA, Rac, cdc42 and JNK are involved in these cellular events (Kühl, Sheldahl et al. 2000; Ishitani, Kishida et al. 2003; van Es, Barker et al. 2003; Veeman, Axelrod et al. 2003; Kohn and Moon 2005; Katoh and Katoh 2007). Wnt ligands show chemical similarity to other secreted glycoprotein ligands, which function through G-protein-coupled receptors (GPCRs), and the Wnt signaling receptors, FZD family receptors, are seven-transmembrane-spanning

receptors that resemble GPCRs (Wang and Malbon 2003). In gonadotrope cells, GnRH has been indicated to target the Wnt signaling effecter, β-catenin, in the regulation of gonadotropins through coordination with Sf-1 (Salisbury, Binder et al. 2007). Since SF-1 is a critical transcription factor, which is also required for the expression of other gonadotropin genes, thus β-catenin was proposed to also engage in mediating the GnRH response to other gonadotropin genes (Salisbury, Binder et al. 2008). GnRH also stimulates several known TCF target genes including Jun, Fra1, and Myc (Wurmbach, Yuen et al. 2001; Yuen, Wurmbach et al. 2002; Nateri, Spencer-Dene et al. 2005; Kikuchi, Kishida et al. 2006), among which Jun is another potential effecter mediating GnRH response to other gonadotropin genes. As Jun subunit is contained in the heterodimers protein AP1, which was known to mediate GnRH response to three gonadotrope signature genes: Cga, Fshβ, and Gnrhr (White, Duval et al. 1999; Norwitz, Xu et al. 2002; Coss, Jacobs et al. 2004; Xie, Bliss et al. 2005). The current knowledge of GnRH-induced targeting of Wnt signaling pathways is illustrated in Figure 1.8.

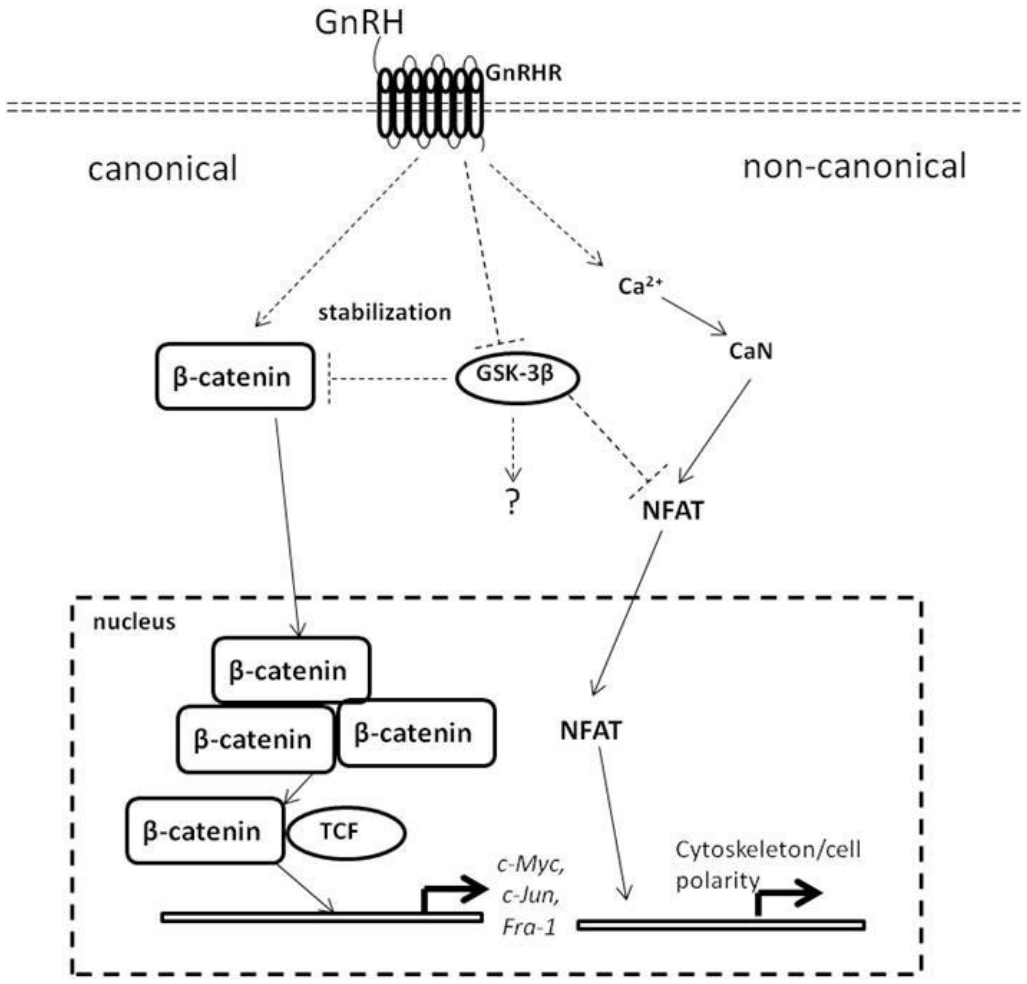


Figure 1.8 Schematic representation of GnRH-induced targeting of Wnt signaling.

GnRH is known to target the canonical Wnt signaling protein, β-catenin, through stimulating β-catenin nuclear accumulation and consequently activating the TCF-dependent transcription. But the mechanism of which is not yet clear, possibly involves GSK-3β. GnRH also regulates the non-canonical pathways through activating Ca^{2+} signaling. This drawing is based on the model proposed by Gardner *et al*. (Gardner and Pawson 2009).

1.3 High-throughput approaches to study regulation of gonadotropins

Because of the complexity of GnRH signaling networks, several groups have applied high-throughput approaches to their study. Most of the earlier studies focused on the transcriptomic level. Extensive studies have been conducted by Sealfon's group. They developed an early gene cDNA microarray technology, which was defined as FMA. They used FMA to characterize the time course from 1 to 6 h of gene responses occurred following activation of the GnRHR in LβT2 cells, and revealed a number of early responsive genes induced by GnRH. Their findings provide information regarding a number of signaling molecules and transcription factors which have potential roles to regulate gonadotropins by GnRH induction (Wurmbach, Yuen et al. 2001; Ruf, Fink et al. 2003; Ruf and Sealfon 2004). Similar study was done by Shawn's group who used GnRH agonist instead of GnRH to treat the LβT2 cells and employed a microarray analysis to identify genes regulated by GnRH agonist at 1 h and 24 h. They found many genes are associated with transcription regulation (Kakar, Winters et al. 2003). Later a microarray study of the genes stimulated by crosstalk of GnRH and activin were carried out. The experiment was designed to find out how GnRH-target genes are affected by activin pretreatment (Zhang, Bailey et al. 2006). The GnRH pulse frequency and amplitude on gonadotropin regulation in LβT2 cells was also studied by microarray in a perifusion system, and a frequency-dependent change in gonadotropin gene expression was identified (Lawson, Tsutsumi et al. 2007). Melamed 's group utilized subtractive hybridization method to screen the altered genes by 8 h GnRH treatment and identified genes crosstalk with estrogen signaling (Luo, Koh et al. 2005). These transcriptomic studies identified a number of signaling molecules and transcription factors, which

provided valuable clues to discover the mechanisms of transcriptional regulation of gonadotropins.

However, the transcriptomic studies can only provide information on mRNA levels, while proteins are dynamic and constantly changing upon both external and internal stimuli (Anderson and Anderson 1998). Transcriptomic results cannot fully represent the state of a biological system such as the protein expression levels, the sub-cellular localization, and the post-translational modifications, etc. Therefore, proteomic studies have become the more useful approaches in the post-genomic era, which has been applied extensively in cancer studies (Roesli, Elia et al. 2006; Batrakou, Kerr et al. 2009; Oeljeklaus, Meyer et al. 2009). So far, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technique is the only proteomic method that has been used for studying GnRH signaling in gonadotrope cells (Feng, Lawson et al. 2008). Feng et al. identified a few proteins involved in mRNA processing, suggesting the proteomic approaches are promising in exploring novel regulatory mechanisms in the regulation of gonadotropins other than transcriptional regulation.

Gel-based technique, such as 2D-PAGE is the earliest developed and most frequently used method for protein profiling, where differentially expressed spots are excised and analyzed by mass spectrometry (MS). However, this method needs to perform a number of replicate experiments to minimize the experimental error (Righetti, Castagna et al. 2004). Besides, the sensitivity of this method is not high, so it is difficult to detect low abundance proteins. Different gel electrophoresis (DIGE) is an alternative approach with the involvement of the fluorescence dyes, used for labeling up to 3 different samples (Lilley, Razzaq et al. 2002). However, the gel-based approach has considerable

limitations. The following groups of proteins will be poorly represented on a 2D gel: proteins with extreme pIs or molecular weight, low abundance proteins, and hydrophobic membrane proteins. Due to the limitations of gel-based approach, quantitative non-2D gel-based technologies have been developed and they have become more and more routine and have the potential to give information about subsets of proteins missing from the 2D gel approach (Lilley and Dupree 2006).

Liquid chromatography (LC)-based approaches allow the combination of stationary and mobile phase for the separation of complex biological samples at the protein or peptide level (Shi, Xiang et al. 2004; Issaq, Chan et al. 2005). For the quantitative profiling of proteins, several stable-isotope labeling methods are developed based on LC-mass spectrometry (MS), among which two frequently used methods are isotope-coded affinity tags (ICAT) and isobaric tag for relative and absolute quantitation (iTRAQ).

1.4 High-throughput proteomic approaches

1.4.1 Mass Spectrometry

Mass spectrometry is a potent analytical technique used to identify unknown compounds, to quantify known compounds, and to elucidate the structure and chemical properties of molecules (Thelen and Miernyk 2012). The fundamental principle of mass spectrometry is to measure the masses of molecules through measuring their mass-to-charge (*m/z*) ratios to confirm the identity of a molecule. A typical mass spectrometer is composed of an ionizer that volatizes and ionizes the analytes, a mass analyzer that measures *m/z* of the ionized analytes, and a detector that records the number of ions at each *m/z* value. The ions are sorted and separated depending on their mass and charge ratio, and then detected and the results are subsequently shown as a readable or graphic display (Horning, Carroll et al. 1977).

Electrospray Ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI) are the two most popular ionization techniques for macromolecules. ESI ionizes the analytes out of a solution and therefore coupled with liquid-based separation tools (Figure 1.9). While, MALDI sublimates and ionizes the samples out of a dry, crystalline matrix through laser pulses (Figure 1.10). MALDI-MS is preferred for analyzing relatively simple peptides mixtures, while integrated liquid-chromatography (LC) ESI-MS is preferred for analyzing complex samples (Aebersold and Mann 2003).

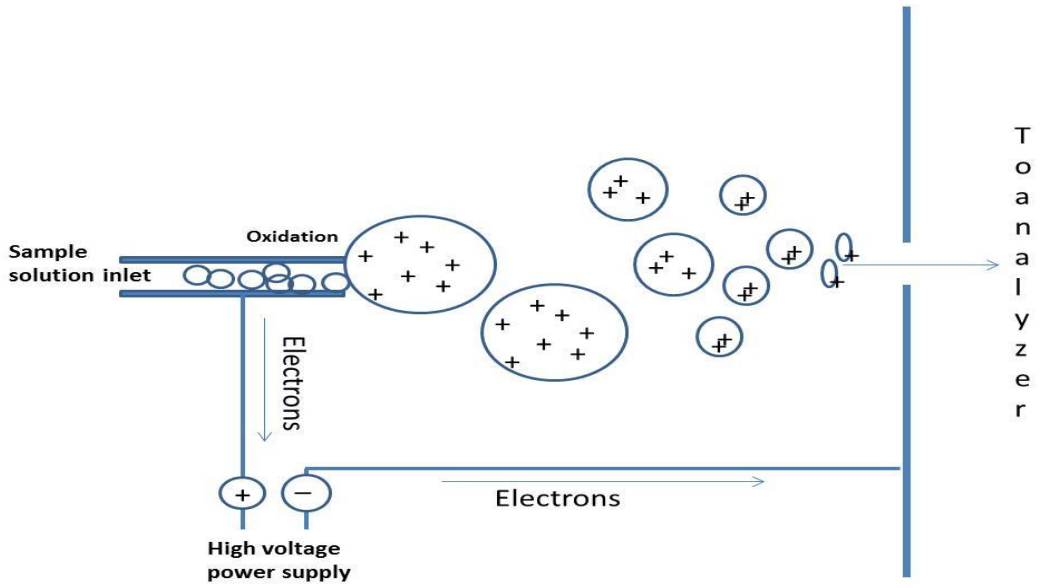


Figure 1.9 Schematic representation of ESI.

A liquid sample solution passes through a nozzle with a very high voltage; the charged liquid in the nozzle becomes unstable as it is forced to hold more and more charges. After reaching a critical point, the liquid blows apart into highly charged droplets, at the tip of the nozzle (Kebarle and Tang 1993).

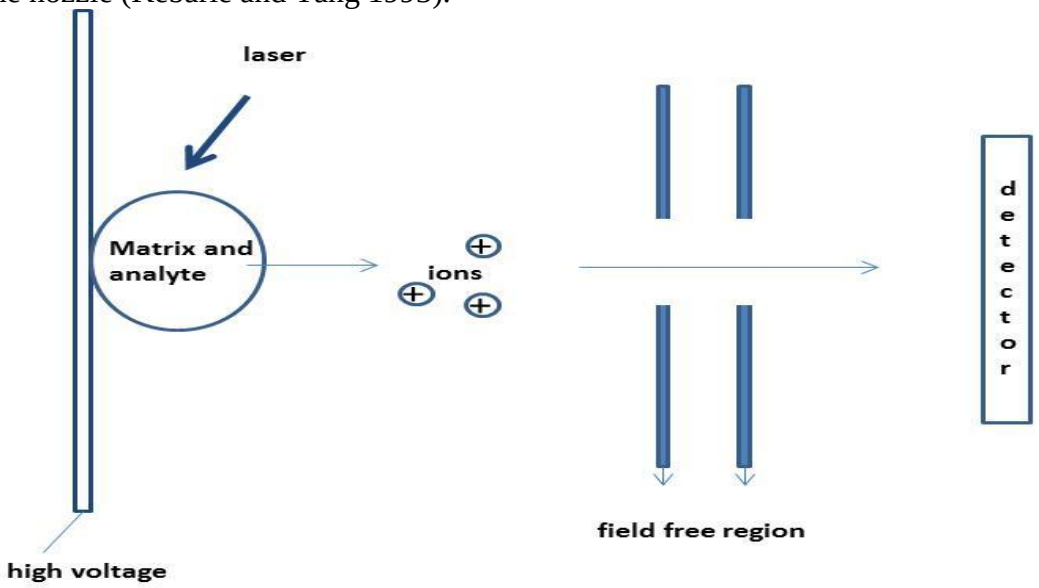


Figure 1.10 Schematic representation of MALDI.

During MALDI, the analyte molecules are ejected by laser pulses from the sample co-crystallized with a matrix, and producing singly charged ions (Karas and Hillenkamp 1988).

1.4.2 ICAT

ICAT labeling reagents have three major components: a biotin affinity tag, which is used to isolate ICAT-labeled peptides; an isotopically coded linker, which can be differentiated by MS; and a reactive group with specificity towards thiol groups of cysteines (Gygi, Rist et al. 1999). Cysteine is a wide coverage but rare amio acid. Around 90 % of mouse proteins have at least 1 cysteine residue; however the occurrence of cysteines in proteins is only 2 % (Miseta and Csutora 2000). The composition of ICAT reagent is demonstrated in Figure 1.11(a). The reagents exist in two forms: heavy and light. In the 1st genration of ICAT reagents, the heavy form contains 8 deuterium and the light form contains no deuterius in the isotopically coded linker, allowing an 8 Da mass difference, which provides discrimination between two samples used for relative quantitation. While in the new generation of ICAT reagents, cleavable ICAT (cICAT) reagents, C^{13} is used to replace deuterium for creating a heavy reagent resulting in a 9 Da mass difference between heavy and light forms. Additionally, the biotin tag is connected to the rest of the molecule through an acid-cleavable linker. Figure 1.11(b) shows the strategy of ICAT experiment. After the two samples are labeled with ICAT reagents, they are combined and digested with trypsin to peptides. The excess reagents are removed through cation exchange chromatography followed by an avidin affinity purification step. The relative quantitation is then determined by the ratio of signal intensities of each peptide pairs (Shiio and Aebersold 2006). In the 1st generation ICAT labeling, the light and heavy ICAT labeled peptides were found to exhibit different retention times on reverse phase HPLC columns. Furthermore, the retention of the biotin tag complicates tandem MS interpretation (Zhang and Regnier 2002). To overcome these problems, the 2nd generation of ICAT was developed. Compared to the 1st generation ICAT reagent, the

new generation allows the co-elution of heavy and light ICAT-labeled peptides, which resolved the problem of retention time difference.

The most advantage of ICAT strategy is that the complexity of the peptide mixture is greatly simplified because only the cysteine-containing peptides are isolated and enriched, thus allowing the detection of the low abundance proteins. However, the specificity of the ICAT reagents for cysteine although contributing to simplify analysis of peptide complex, is also one of the drawbacks as this strategy can only be applied to cysteine-containing proteins. While many regulatory proteins, especially those with post-translational modifications (PTMs), are overlooked by this technique (Zieske 2006).

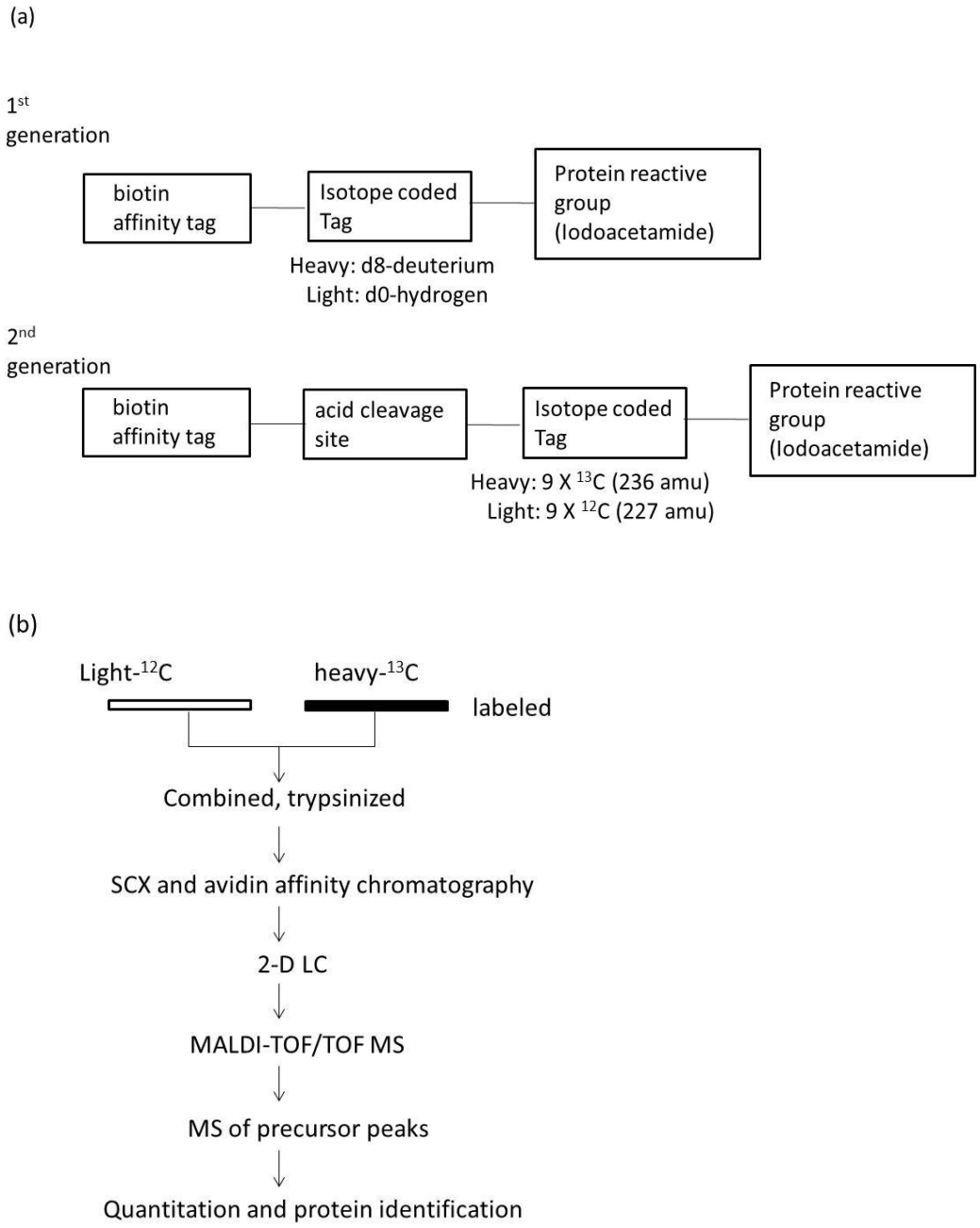


Figure 1.11 The ICAT strategy for proteomics experiment.

(a) Structure of the ICAT reagents; (b) Flowchart of ICAT-labeling based experiment.

1.4.3 iTRAQ

iTRAQ labeling is a more recently developed proteomic approach by Darryl Pappin and colleagues at Applied Biosystems in 2004 (Coss, Jacobs et al. 2004). iTRAQ reagents are a group of amine-specific isobaric reagents, which can be used to identify and quantify up to eight different samples simultaneously. Each isobaric tag is composed of a charged reporter group, a peptide reactive group, and a neutral balance portion to keep an overall mass of 145 for 4-plex iTRAQ reagents or 305 for 8-plex iTRAQ reagents (Figure 1.12 (a)). The reporter group is unique to each of the 4 (114--117) or 8 (113-121) reagents, which give rise to unique reporter ions upon MS/MS fragmentation for quantitation. The peptide reactive group is able to react with all primary amines, including the N-terminus and the ε-amino group of the lysine side chain. Thus this method enhances peptide coverage for any given protein while keeping other important information such as PTMs (Zieske 2006). The general workflow is described in Figure 1.12 (b) for 4-plex iTRAQ analysis. Individual samples are reduced, alkylated and trypsin-digested. The resulting peptide mixture is subsequently parallel-labeled with iTRAQ reagents provided by the multiplex set. The labeled peptides are then combined and subjected to LC-MS/MS analysis. The development of the iTRAQ strategy expands the application of comparative proteomics to numerous experimental approaches such as time-course analysis (Petti, Thelemann et al. 2005), affinity pull down analysis (Wu, Warren et al. 2010), and disease markers identification (DeSouza, Diehl et al. 2005). In addition, as most of the peptides in a sample are conformable to iTRAQ labeling, there will be less information loss compared to ICAT labeling. Hence, this strategy increases the chance to identify the proteins containing PTMs. Finally, the multiplexity of the reagents provides the additional statistical validation within any given experiment.

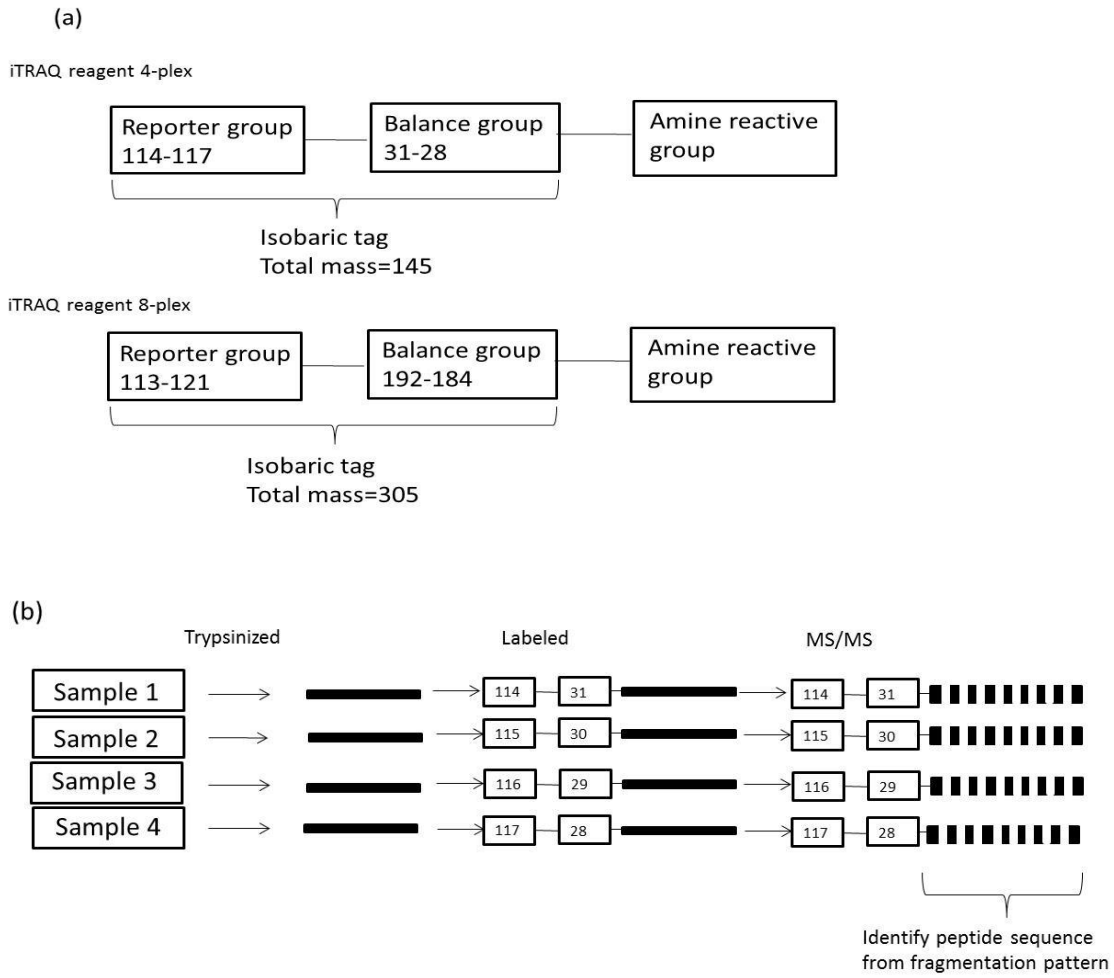


Figure 1.12 The iTRAQ strategy for proteomics experiment.

(a) Structure of the iTRAQ reagent 4-plex (total mass=145) and 8-plex (total mass=305);
(b) Schematic representation of 4-plex iTRAQ-labeling based experiment.

1.4.4 Bioinformatics analysis of gene function

The development of high-throughput profiling technologies gives rise to the development of knowledge-based pathway analysis, which is able to extract the meaning from a long list of genes and proteins. The main idea of the system-wide data analysis approach is a knowledge base that contains extracted literature information used for functional data analysis and network generation (Ganter, Zidek et al. 2007). A number of knowledge bases help researcher to investigate the biological processes, cellular components, and molecular functions related to their experimental dataset.

Ingenuity pathway analysis (IPA) is a commercial pathway analysis tool. It was designed to meet the requirement of a comprehensive application purpose from the fundamental core analysis to more specific metabolic, biomarker, and toxicity analysis (Khatri, Sirota et al. 2012). Search Tool for the Retrieval of Interacting Genes (STRING) http://string-db.org/ is an open source pathway analysis tool. It allows the analysis and search for comprehensive functional association between the proteins. The functional association is deduced by both experimental and predicted information. And most importantly, it is provided with a confidence score, and supplemental information such as protein domain and 3D structure. All these above information can be obtained within one stable and consistent identifier space (Szklarczyk, Franceschini et al. 2011). Database for Annotation, Visualization and Integrated Discovery (DAVID) gene functional classification tool http://david.abcc.ncifcrf.gov/ is another useful open source program to analyze gene function. It uses a novel algorithm, to compile a list of genes or associated biological terms into biological modules, which refer to the organized classes of related genes or biology. This organization is achieved by looking for the complex biological co-

occurrences occurring in multiple sources of functional annotation. It is a powerful tool to group the functionally associated genes and terms into a manageable number of biological modules, which can then be efficiently used to interpret the list of genes in a network context (Huang, Sherman et al. 2007).

Although these bioinformatics approaches are greatly helpful in extracting and explaining the underlying biology for high-throughput molecular measurement, the limitation of these approaches also needs to be addressed. One obvious problem is that the true pathway topology is quite cell-contextual dependent. For example, GnRH induced MAPK activation has been found varied among different pituitary cells (Dobkin-Bekman, Naidich et al. 2006). Recently, an open source knowledgebase of the GnRHR signaling in the LβT2 cells was developed by using 106 relevant publications (Fink, Pincas et al. 2010). Compared to the IPA pathway analysis, this pathway analysis method more specifically depicted the GnRH signaling in gonadotrope cell. However, due to the limited space on the pathway map, this knowledgebase doesn't include the modification events, which are considered to play pivotal roles in regulating a wide range of biological processes.

1.5 Murine gonadotrope cell lines

Studies of the regulation of gonadotropin genes and GnRH signaling have been the subject of intense experimental investigation for the past 25 years, by means of both *in vivo* and *in vitro* approaches. *In vitro* models have mainly consisted of pituitary primary cultures, heterologous cell systems, and the gonadotrope cell lines αT3-1 and LβT2 (Fink, Pincas et al. 2010). Because pituitary gonadotropes account for only 5-10% of the

anterior pituitary cell population, using primary cultures as a model for the study of gonadotrope-specific events is challenging.

The gonadotrope cell lineage originates in the ventral part of the embryonic pituitary. The expression of the α-subunit gene is observed at approximately embryonic day 11.5 (e11.5) of gestation in the mouse (Japon, Rubinstein et al. 1994) followed by the expression of the individual β-subunits of LH on e16.5 and FSH on e17.5. The immortalized gonadotrope cell lines αT3-1 and LβT2 were obtained by directed oncogenesis in transgenic mice. The αT3-1 cell line was generated through targeting expression of the SV40 large T antigen using the regulatory region of the human glycoprotein hormone α-subunit gene (Windle, Weiner et al. 1990), and the LβT2 cell line was, in turn, generated through targeting oncogenesis with the 5'-flanking region of the LHβ gene (Alarid, Windle et al. 1996). The αT3-1 cell line represents an immature gonadotrope that is not fully differentiated and expresses the glycoprotein hormone α-subunit, responds to GnRH, but does not express the LHβ and FSHβ subunits (Windle, Weiner et al. 1990). In contrast, the LβT2 cell line displays the characteristics of a fully differentiated gonadotrope: in addition to α-subunit, it expresses LHβ, FSHβ, GnRHR, activin, inhibin, follistatin and steroid receptors (Windle, Weiner et al. 1990; Alarid, Windle et al. 1996; Turgeon, Kimura et al. 1996; Graham, Nusser et al. 1999; Hammer, Krylova et al. 1999) (Figure 1.13). Consequently, these two cell lines represent useful model for the study of FSHβ and LHβ gene-specific transcription mechanisms at both basal and GnRH-regulated levels.

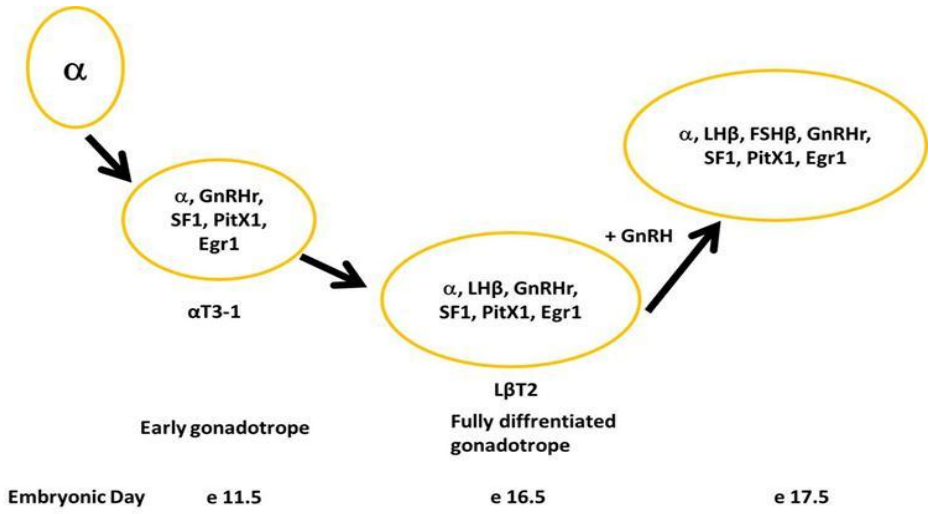


Figure 1.13 Schematic representation of the gonadotrope cell lineage development in mouse.

The initiation of anterior pituitary differentiation is marked by the expression of the glycoprotein hormone α subunit gene. The emergence of mature gonadotrope happens in two stages, with the expression of LHβ and FSHβ subunit genes on e16.5 and e17.5, respectively. These temporal and distinct stages of differentiation were applied by target oncogenesis in transgenic mice to generate immortalized immature and mature pituitary αT3-1 and LβT2 cell lines (Alarid, Windle et al. 1996).

Chapter 2. Aims of study

GnRH is the first key hormone of reproduction. The binding of GnRH to its cognate type I receptor initiates diverse signaling cascades. Among them, the MAPK signaling cascades are the most studied and characterized. The GnRH-induced MAPK downstream signaling enables the gonadotrope to response to GnRH and translates the signals into differential gonadotropin synthesis and secretion.

Through utilizing high-throughput transcriptomic approaches, a number of transcription factors have been revealed to mediate GnRH-induced transcriptional regulation of gonadotropins. However, the gene regulations not only occur at the transcriptional level but also at the post-transcriptional, translational and posttranslational levels. Therefore, we would like to perform high-throughput proteomic studies in order to identify those factors involved in the regulations after transcription. Besides MAPK signaling cascades, recent studies indicate that GnRH-induced gonadotropin production may also be regulated through other signaling pathways. However, the current knowledge in this field is still rather limited. Only a few genes have been identified, which are insufficient to elucidate an integrative map. So there is a need to identify more genes/proteins in order to elucidate those signaling pathways. Furthermore, nowadays the study of GnRH signaling is no longer staying at a static status, but rather moving to the GnRH pulse frequency induced differential regulation of gonadotropins. How the gonadotropins decode the GnRH frequency is still a mystery.

The main aims of the project are:

1) To identify low abundance signaling molecules. Whole cell lysate from mock-treated and GnRH-treated LβT2 cells were compared by cICAT-based proteomics experiment.
2) To functionally validate the candidates identified by cICAT proteomic profiling, and propose signaling pathways based on the findings.
3) To identify the proteins involved in posttranscriptional, translational and posttranslational regulation of gonadotropins. Nuclear cell lysate from mock-treated and GnRH-treated LβT2 cells were compared by iTRAQ-based proteomics experiment.
4) To functionally validate the most prominent proteins identified by iTRAQ proteomic profiling.
5) To identify the proteins induced by different GnRH frequency treatment, and predict their functional correlation with the GnRH frequency.

Chapter 3. Materials and methods

3.1 Cell culture

3.1.1 Growth condition

LβT2 cells (generously provided by Dr. P. Mellon, University of California, San Diego, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 % certified fetal calf serum, 10 mM HEPES (pH 7.4), 100 U/ml penicillin and 100 µg/ml streptomycin, which were purchased from Invitrogen (Carlsbad, CA, USA), and were incubated at 37 °C under 5 % CO_2.

3.1.2 GnRH treatment of cells

LβT2 cells were first starved 12 h in Modified Eagle Medium (MEM; Gibco) starvation medium, supplemented with 10 mM HEPES (pH 7.4), 100 U/ml penicillin and 100 µg/ml streptomycin, 1 % Essential Amino Acid (Gibco), 1 % Sodium Pyruvate (Hyclone), and then treated with either 100 nM GnRH ([D-Ala6]-LHRH; Sigma; dissolved in DMSO) or mock (DMSO)-containing MEM starvation medium for either 4 h or 30 min.

3.1.3 Transient transfection

Transfection was carried out at 70-80 % confluence after cells were plated overnight. LβT2 cells were transfected using either GenePORTER 2 (Gene Therapy Systems) or Lipofectamine 2000 (Invitrogen) Transfection Reagent. The optimized transfection conditions were shown in Table 3.1. For GenePORTER2 transfection, the hydrated GenePORTER 2 reagent was first mixed with serum-free MEM and the DNA plasmids were incubated with DNA diluent at room temperature (RT) for 5 min, and then they were mixed together and further incubated for 10min at RT before they were added to the cells. For Lipofectamine transfection, both the Lipofectamine 2000 reagent and the DNA plasmids were mixed with serum-free MEM in separate eppendorf tubes. After 5 min

incubation at RT, the DNA solution was added to the diluted Lipofectamine2000 reagent and further incubated for 20 min at RT before they were applied to the cells.

Table 3.1 Optimized GenePORTER2 and Lipofectamine 2000 transfection conditions.

Culture Vessel	96-well	24-well	6-well	60-mm	100-mm
DNA (ul)	0.1-0.5	2	6	8	12
DNA diluents (ul)	0.5-2.5	50	150	200	300
Gene PORTER2(ul)	2.5	10	30	40	60
SFM(MEM) (ul)	2-10	40	120	160	240
DMEM Culture Medium (ul)	100	250	1000	2500	5000

Culture Vessel	6-well
DNA (ul)	4
Lipofectamine 2000 (ul)	9
SFM(MEM) (ul)	125
DMEM Culture Medium (ul)	100

3.2 Sample preparation and protein quantitaion

3.2.1 Sample preparation for ICAT study

3.2.1.1 Whole cell lysate preparation

The mock-treated and GnRH-treated LβT2 cells were rinsed with PBS for 3 times, and were detached from culture dishes by 1 ml of 0.5 mM EDTA. The cells were blew off from culture dishes using PBS, and collected by centrifugation (Eppendorf 5810R) at 3500 rpm for 10 min at 4°C. The packed cell volume (pcv) was measured and the cells were resuspended in ICAT lysis buffer, which is composed of 0.5 M triethylammonium bicarbonate buffer (TEAB) (Sigma), 1 % SDS with supplement of cocktail proteinase inhibitors (Roche) and phosphatase inhibitors (Sigma), to a final volume of 4 pcv. The cells were swelled on ice for 10 min, and homogenized with 15 up-and-down strokes in a glass Dounce homogenizer (Sigma). The cell lysate was heated at 97 °C for 10 min, and the cell debris was removed by ultracentrifugation (Beckman Optima^{TM} TLX) at 25000 rpm for 30 min at 4 °C, and the supernatant was stored at -80 °C for further application.

3.2.1.2 RC-DC quantitation

The protein concentration was determined by RC-DC quantitation (Biorad). All the procedures were done following the protocol provided by the RC-DC quantitation kit. BSA stock solution was prepared by reconstitute BSA powder (Sigma) in ICAT lysis buffer at 2 mg/ml. BSA standard curve was plotted using 5 consecutive dilutions (0, 0.2, 0.4, 0.8, 1.2, 1.5 mg/ml) of the BSA standard in ICAT lysis buffer. Three replicates were prepared for each dilution. Total protein lysate was diluted 8-fold using ICAT lysis buffer, and the concentration was determined based on the equation derived from the BSA standard curve.

3.2.2 Sample preparation for iTRAQ study

3.2.2.1 Separation of nuclear and cytoplasmic proteins

All the buffers mentioned in this section were listed in Table 3.2. The cell pellet was collected as described in 3.2.1.1, and was rapidly resuspended in 5 pcv of hypotonic buffer, and centrifuged at 4500 rpm for 5 min at 4 °C. The supernatant was discarded and the packed cells were resuspended again in 3 pcv of hypotonic buffer. After swelling on ice for 10 min, the cells were homogenized with 15 up-and-down stokes in a glass Dounce homogenizer and pelleted down at 6000 rpm for 15 min at 4 °C. The supernatant was saved for cytoplasmic extract preparation, and the pellet was used for further nuclear protein extraction.

Cytoplasmic proteins were extracted from the supernatant by adding 0.11 pcv of 10× cytoplasmic extract buffer. The mixture was mixed thoroughly by vortexing and pelleted down by ultracentrifugation at 50,000 rpm for 1 h at 4 °C. The supernatant was saved for further desalting.

The nuclei pellets were resuspended in low-salt buffer equal to ½ packed nuclear volume (pnv), and drop-wisely mixed with ½ pnv of high-salt buffer. The nuclear protein was extracted by 10 s vortexing every 5 min for up to 30 min. The extracted nuclei were pelleted down by ultracentrifugation at 25,000 rpm for 30 min at 4 °C. The supernatant was saved for further desalting.

Table 3.2 Buffers used in separation of nuclear and cytoplasmic proteins.

Buffers	Component
Hypotonic buffer	10 mM HEPES, pH 7.9 at 4 °C 1.5 mM $MgCl_2$ 10 mM KCl 0.5 mM DTT cocktail proteinase inhibitors phosphoproteinase inhibitors
Low salt buffer	20 mM HEPES, pH 7.9 at 4 °C 25 % glycerol 1.5 mM $MgCl_2$ 0.2 mM EDTA 0.5 mM DTT cocktail proteinase inhibitors phosphoproteinase inhibitors
High salt buffer	20 mM HEPES, pH 7.9 at 4 °C 25% glycerol 1.5 mM MgCl2 800 mM KCl 0.2 mM EDTA 0.5 mM DTT cocktail proteinase inhibitors phosphoproteinase inhibitors
Cytoplasmic extract buffer, 10×	300 mM HEPES, pH 7.9 at 4 °C 1.4 M KCl 30 mM $MgCl_2$ cocktail proteinase inhibitors phosphoproteinase inhibitors

3.2.2.2 Desalting

Sample desalting was carried out using the centrifugal filter devices YM-3, Microcon (Millipore). The raw samples from cytoplasmic and nuclear extraction were added to the sample reservoirs together with the iTRAQ lysis buffer, 0.5 M TEAB + 0.1 % SDS. The high salt buffer which was contained in the original samples was gradually replaced by repeated filling the sample reservoir with iTRAQ lysis buffer and continuous centrifugation at 14,000 g, 4 °C.

3.2.2.3 Bradford quantitation

The protein concentration was determined by Bradford quantitation method (Biorad). All the procedures were done following the protocol provided by the Bradford quantitation kit. BSA stock solution was prepared by dissolving BSA powder (Sigma) in iTRAQ lysis buffer at 20 mg/ml, and further diluted to 1 mg/ml in H_2O. BSA standard curve was plotted through 6 consecutive dilutions (0, 100, 125, 150, 175, 200, 225 µg/ml) of BSA standard. Three replicates were prepared for each dilution. Protein samples were diluted 20-fold with H2O, and their concentrations were determined based on the equation derived for the BSA standard curve.

3.2.3 Calibration of protein quantitation by silver staining

After quantitation by either RC-DC or Bradford method, protein concentrations were further confirmed with silver staining. 1 µg of each sample was resolved with the SDS-PAGE gel and silver-stained. The recipe and procedure of silver staining steps were shown in Table 3.3. The protein concentration was then finely adjusted according to the silver staining result.

Table 3.3 Recipe and procedure of silver staining.

Buffers (for 1pcs gel)	Component (for 25ml solution)
Fixing buffer	50 % methanol 12 % acetic acid 12.5 ul of 37 % formalin 55 rpm RT 30 min
Washing buffer 1	35 % ethanol 55 rpm 3*20 min
Sensitizing buffer	0.02 % sodium thiosulphate 80 rpm 2 min
Washing buffer 2	H_2O 55 rpm 3*5 min
Silver buffer	0.2 % silver nitrate 19 ul of 37 % formalin 80 rpm 20 min
Washing buffer 3	H_2O 55rpm 2*1min
Developing buffer	6 % sodium carbonate 0.0004 % sodium thiosulphate 12.5 ul of 37 % formalin 110 rpm until band appears
Stopping buffer	25 mM Sodium EDTA 110 rpm 20 min
Washing buffer 4	H_2O 55 rpm 3*5 min Store at 4 °C

3.3 Quantitative proteomics using Stable-Isotope labeling technologies

3.3.1 Cleavable Isotope Coded Affinity Tag (cICAT) labeling

cICAT labeling and processing of the samples were carried out according to the kit instruction (AB SCIEX). After quantitation, 100 μg of proteins from each of the control (labeled with cICAT light) and GnRH-treated cell lysate (labeled with cICAT heavy) were reduced with 1.25 mM Triscarboxyethylphosphine (TCEP), and subsequently labeled with the isotopic light- and heavy- forms of the cICAT reagents respectively for 2 h at 37 °C in the dark. Each pair of heavy- and light-cICAT-labeled proteins were then combined and trypsinized at 37 °C for 16 h. Upon completion of *in situ* digestion, the digested peptide mixture was passed through a strong-cation exchange cartridge, and then enriched with an avidin affinity cartridge. The cICAT-labeled peptides were then dried by a SpeedVac concentrator (Savant) and re-dissolved in cleaving reagents and incubated at 37 °C for 2 h. After the removal of biotin, peptides were desalted using Sep-Pak C18 cartridges (Waters) followed by lyophilization before being reconstituted for 2-Dimensional liquid chromatography (2-D LC).

3.3.2 Isobaric Tag for Relative and Absolute Quantification (iTRAQ) labeling

8-plex iTRAQ labeling of each sample was performed as described in manufacturer's protocol (AB SCIEX). A total of 100 μg proteins from each mock-treated and GnRH-treated sample were reduced with 5 mM TCEP at 60 °C for 1 h, and then alkylated with 10 mM methyl methanethiosulfonate (MMTS) for 10 min. After cysteine blocking, each sample was diluted to 0.05% (w/v) SDS before trypsin digestion at 37 °C for 16 h. Each digested sample was then labeled for 1 h with one of the eight isobaric amine-reactive tags. These 8-plex iTRAQ-labeled samples were then pooled and passed through a

strong-cation exchange (SCX) cartridge as recommended by the manufacturer (AB SCIEX). The eluate was subsequently desalted using a Sep-Pak cartridge (Waters). Finally each sample was lyophilized and reconstituted for 2-D LC analysis.

3.3.3 2-D LC and Mass Spectrometry (2D-LC MS/MS)

Each of the cICAT and iTRAQ-labeled peptide mixtures was reconstituted in 2 % (v/v) acetonitrile (ACN) with 0.05 % (v/v) trifluoroacetic acid (TFA) and after centrifugation the supernatant was separated using an Ultimate^{TM} dual-gradient LC system (Dionex-LC Packings) equipped with a Probot^{TM} MALDI spotting device. In the first dimensional separation, the labeled peptide mixture was first injected into a SCX column (300 μm i.d., × 15 cm, packed with 10 μm POROS 10S; Dionex-LC Packings) at a flow rate of 6 μl/min. Nine fractions were obtained using step gradients of mobile phase B buffer: unbound, 0-5, 5-10, 10-15, 15-20, 20-30, 30-40, 40-50, 50-100 % of B. In the second dimensional separation, the eluted fractions were captured alternatively onto two PepMap^{TM} trap columns (300 μm i.d., × 1 mm, packed with 5 μm C18, 100 Å; Dionex-LC Packings) and washed with 0.05% TFA followed by gradient elution to a reverse-phase (RP) Monolithic Capillary column (200 μm i.d., × 5 cm; Dionex-LC Packings). The LC fractions were mixed with matrix-assisted laser desorption/ionization (MALDI) matrix solution at a flow rate of 5.4 μl/min via a 25 nl mixing-tee (Upchurch Scientific) before they were spotted in 28 X 44 spot arrays on 123 × 81 mm Opti-TOF LC-MALDI inserts (AB SCIEX) using a Probot Micro Precision Fraction collector (Dionex-LC Packings) at a speed of 1 spot per 5 sec. The buffers used in 2D-LC MS/MS were shown in Table 3.4.

Table 3.4 Buffers used in 2-D LC MS/MS.

Buffers	Component
Mobile phase A (1st-dimensional)	5 mM KH_2PO_4, pH 3 5% ACN
Mobile phase B (1st-dimensional)	5 mM KH_2PO_4, pH 3 5 % ACN 500 mM KCl
Mobile phase A (2nd-dimensional)	2 % ACN 0.05 % TFA
Mobile phase B (2nd-dimensional)	80 % ACN 0.04 % TFA
MALDI matrix solution	7 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) 130 μg/ml ammonium citrate in 75 % ACN

3.3.4 Tandem Mass Spectrometric analysis

The MS and MS/MS of ICAT samples were analyzed using an ABI 4800 Proteomics Analyzer (AB SCIEX) with a MALDI ionization source and TOF/TOF optics operating in MS-positive reflector mode. Laser intensity was set to 4000 for MS and 4300 for MS/MS acquisition. For MS analysis, typically 1000 shots were accumulated for each sample well. Tandem MS/MS analyses were performed using nitrogen at collision energy of 2 kV and a collision gas pressure of ~1 X 10^{-6} Torr. 2000-5000 shots were combined for each spectrum depending on the quality of the data.

The MS analysis of iTRAQ samples were analyzed using an ABI 5600 TripleTOF analyzer (QqTOF; AB SCIEX) operating in information dependent mode. The mass range of 400-1800 m/z with 250 ms accumulation time for each spectrum was used for selecting the precursor ions. From each MS spectra, a maximum of 20 precursors were selected per cycle for MS/MS analyses with 100 ms minimum accumulation time for each precursor and dynamic exclusion for 15 s. Tandem MS was recorded in a high sensitivity mode with both rolling collision energy and iTRAQ reagent collision energy adjustment on.

3.3.5 Peptide and protein identification

Protein identification and ICAT quantification were performed with the GPS Explorer™ software Ver. 3.6 (AB SCIEX) using the MASCOT search engine (V2.1; Matrix Science) and normalized against median ratio obtained from all the cICAT peptide pairs detected in one sample. The ratios were calculated based on the cluster areas of the heavy-cICAT labeled peptides and those of the light-cICAT labeled peptides. The International Protein Index (IPI) mouse database (v3.75) was used for the search of tryptic peptides.

Protein identification and iTRAQ quantification were performed with ProteinPilot™ Software 4.2 (AB SCIEX) using the Paragon™ algorithm for the peptide identification, which was further processed by Pro Group™ algorithm that allows the isoform-specific quantification to differentiate the expression between various isoforms. The user defined search parameters were as follows: (1) Sample type, iTRAQ 8plex (Peptide labeled); (2) Cysteine alkylation, MMTS; (3) Digestion, Trypsin; (4) Instrument, TripleTOF5600; (5) Special factors, None; (6) Species, Mus musculus; (7) ID focus, Biological modifications; (8) Database, ipi.MOUSE.v3.87.fasta; (9) Search effort, Thorough; (10) FDR Analysis: Yes; (11) User modifies parameter files: No. The peptide list obtained from ProteinPilot™ was exported to Microsoft Excel for further analysis. The peptides for quantitation were automatically selected by the software, by removing those with shared MS/MS, low confidence, weak signal or discordant peptide type, to calculate the reporter peak area, error factor (EF) and *p* value. The resulting data were set as auto bias-corrected to exclude the artificial errors caused by unequal mixing of the individual labeled samples.

3.3.6 Determination of the significant cut-off threshold for fold-change

The significant cut-off threshold for ICAT dataset was determined using the standard deviation (S.D.) method as described by Tan *et al*. (Tan, Tan et al. 2008). Thus, the cut-off threshold for cICAT labeled peptides was set as >1.3 for up-regulated proteins and <0.77 for down-regulated proteins.

The significant cut-off threshold for iTRAQ dataset was determined using a more stringent method, biological replicate method, which has been described by Gan *et al*. (Gan, Chong et al. 2007). Briefly, the numbers of the proteins fell in each of the cumulative percentage coverage (0 % to 100 %) were plotted against the corresponding percentages of variation (5 % to 80 %). By doing this, around 48% variation was found to corresponding to 88% coverage of data, therefore the final cutoff was fixed at 1.5 fold (±50 % variation), which corresponds to >1.5 for up-regulation and <0.67 for down-regulation.

3.3.7 Estimation of false positive rate to determine cut-off score

For ICAT dataset, a randomized database created using IPI mouse database Version 3.75 was also used to search the cICAT-labeled peptides. The False Positive Rate (FDR) was calculated by comparing the search results from the randomized database versus the actual database. The minimum ion score confidence interval (C.I.) % was set as the value at which no more than 5 % FDR was achieved. Based on this cut-off threshold, all the proteins identified from the random database search were single-peptide matched. For single-peptide matched proteins, only those with ion score C.I. % greater than the highest C.I. % attained from the random database search were selected as significant. For iTRAQ

dataset, the cut-off for unused proteins score was calculated as ≥ 1.3, corresponding to 95 % C.I., was used as the first quantification criteria.

3.3.8 Bioinformatics analysis

Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com) and Core Analysis module was selected to analyze the top biological processes as well as molecular and cellular functions. The reference set and parameters for IPA were shown as follows: (1) Reference set, Ingenuity Knowledge Base (Genes only); (2) Relationship to include, Direct and Indirect, Includes Endogenous Chemicals; (3) Filter Summary, Consider only relationships where confidence=Experimentally Observed OR High (predicted).

The open source program DAVID (http://david.abcc.ncifcrf.gov/) was used to analyze the top-ranked molecular functions among a given list of proteins using the *mouse* genome as reference. After uploading all the protein IDs, the IPI_ID was chosen as the identifier, and Gene list was chosen as the list type, and the list was submitted to the software server. Under the “Functional _categories” panel, “SP-PIR_KEYWORDS” was selected for the functional characterization analysis. The GO was reported with a Benjamini (FDR corrected) *p*-value <0.05.

The open source STRING (http://string-db.org/) was used to identify the top interaction networks among the significantly altered proteins. After uploading all the protein IDs into the software server, the "GO enrichment" panel was selected and the top terms were looked for under each of the biological processes, molecular functions and cellular components and subsequently the protein IDs were exported from each selected terms.

And then the protein IDs from each top terms were uploaded again and the actions view was selected to check the interaction maps including nodes (individual proteins) and edges (biological relationship between nodes).

3.4 RT-PCR analysis

3.4.1 RNA extraction and reverse transcription for cDNA synthesis

RNA isolation from LβT2 cells was performed using Trizol (Invitrogen) according to the manufacturer's instruction. First the cells were incubated with the Trizol reagent (1 ml of Trizol for 6-well plate, 3 ml of Trizol for 60 mm plate) for 5 min at RT to allow dissociation of the nucleoprotein complex, and then 0.2 ml of chloroform (Merck) per 1 ml of Trizol reagent was added for homogenization. After vigorous vortexing for 15 sec, the mixture was incubated for 3 min at RT. The RNA aqueous phase was separated by centrifugation at 12,000 g for 15 min at 4 °C, and transferred by pipetting to a new tube. The RNA present in the aqueous phase was further precipitated by 0.5 ml isopropanol (Sigma) per 1 ml Trizol reagent followed by centrifugation at 12,000 g for 10 min at 4 °C. RNA pellet was washed with 1 ml of 75% ethanol and collected by centrifugation at 7,500 g for 5 min at 4 °C. The purified RNA was then dried and re-dissolved in nuclease-free water (GIBCO).

Reverse transcription was performed in a 20 µL reaction. 2 µg of RNA obtained from Trizol extraction was first mixed with 1 µL of 500 µg/ml Oligo $(dT)_{12-18}$ (NEB) and 1 µL of 10 mM dNTPs mix (promega), heated to 65 °C and quickly chilled on ice. And then 4 µL of 5X M-MLV Reverse transcriptase buffer and 1 µL of transcriptase (Promega) were

added and the reaction was initiated at 42 °C for 80 min followed by inactivation at 70 °C for 15 min.

3.4.2 Semi-quantitative RT-PCR

Of the 20 µl cDNA obtained, 1 to 2 µl was used for a semi-quantitative PCR with specific primers (Table 3.5). The PCR reaction mixture and the parameters for PCR were shown in Table 3.6. PCR products were then analyzed on 1.5 % agarose gel (1st Base).

Table 3.5 Primers used for RT-PCR and Real-time PCR analysis.

Primers	Sequence (5' to 3')
mFSHβ gene F	GACAGCTGACTGCACAGGAC
mFSHβ gene R	GTTAGATAACCAACAGTATG
mLHβ gene F	GCCTGTCAACGCAACTCTGG
mLHβ gene R	CAGGCCATTGGTTGAGTCCT
mGnRHR gene F	CCACAGTGGTGGCATCAGGCCTTC
mGnRHR gene R	TAGCGTTCTCAGCCGAGCTCTTGG
60s RP gene F	ATGGTCCAGCGTTTGACATA
60s RP gene R	TTTCCCAACCTTCTTGGTGT
ACTN4 RT F	AGCAATCACATCAAGCTGTCG
ACTN4 RT R	CCACACTCATGATCCGGTTG

Table 3.6 RT-PCR reaction mixture and cycling parameters.

Components	Volume (μL)
cDNA template	1˜2
10 X Dynazyme buffer (Finnzyme)	2.5
10 mM dNTPs	0.5
Forward primer (10 μM)	2.5
Reverse primer (10 μM)	2.5
Dynazyme (2 U/μL, Finnzyme)	0.5
Nuclease-Free Water to final volume	25

Step	Cycle	Temperature (°C)	Time
1	1	95	3 min
2	25-35	95 Tm-5 72	20s 30s 30s
3	1	72	5 min
4	1	4	∞

3.4.3 Quantitative real-time RT-PCR

Real-time PCR was performed using Power SYBR PCR master mix (Applied Biosystems) with an ABI PRISM 7000 Sequence Detection System instrument (Applied Biosystems). Primers for real-time PCR are the same as those listed in Table 3.5. The samples were first warmed to 50 °C for 2 min, and then heated to 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative changes in mRNA levels were calculated using the ΔΔCt algorithm. Each value is representative of three replicates.

3.5 Plasmid construction

3.5.1 Expression plasmids

The expression plasmids for full length and truncated ACTN4 were generated by PCR amplification and subsequently digested and ligated with the indicated vector. The corresponding primer sets and vectors for generating each expression plasmid were shown in Table 3.7. For insert PCR amplification, a 50 μL PCR reaction was set up as shown in Table 3.8. The produced insert(s) were then purified by PCR purification kit (Promega) and eluted in 50 μL H_2O. 5 μL of insert and 0.5 μg of vector were mixed together in a 20 μl digestion system (Promega). After being digested with the individual restriction enzymes, whose site is carried by each primer, at 37 °C for 2 h, the mixture was purified by PCR purification kit, and finally eluted in 17 μl H_2O. After adding the T4 DNA ligation buffer and ligase (Promega), the mixture was incubated at 25 °C for 2 h. 5-20 μL of resulting ligation product was used for transformation followed by PCR screening. Cacybp over-expression plasmid was generated as previously described (Ghosh, Yu et al. 2011).

Table 3.7 Primer sets for PCR amplification of inserts for expression plasmids.

Target Gene	Accession Number	Expression vector	Restriction sites	Forward primer sequence (5' to 3') Reverse primer sequence (5' to 3')
ACTN4	NM_021895.2	pXJ40-FLAG	HindIII XhoI	CCCAAGCTTATGGTGGACTACCACGCAGC
				CCGCTCGAGTCACAGGTCGCTCTCCCCAT
ΔCaM-ACTN4	NM_021895.2	pXJ40-FLAG	HindIII XhoI	CCCAAGCTTATGGTGGACTACCACGCAGC
				CCGCTCGAGTCAAATGAAGGCTTGGAAGGTC
Cacybp	NM_001007214.1	pXJ40	XhoI PstI	CCGCTCGAGATGGCTTCAGAAGAGCTA
				AACTGCAGTCAAAATTCCGTGTCTCCTTTG

Table 3.8 Components of PCR amplification of inserts for expression plasmids.

Components	Volume (μL)
cDNA template	5
5 X GoTaq® Flexi buffer (Promega)	10
PCR Nucleotide Mix (10 mM each)	1
$MgCl_2$ solution (25 mM)	4
Forward primer (10 μM)	5.0
Reverse primer (10 μM)	5.0
GoTaq® polymerase (5 U/μL, Promega)	0.25
Nuclease-Free Water to final volume	50

3.5.2 Luciferase vectors

LHβ promoter was created by ligating 1300 bp of the proximal murine LHb gene promoter into pGL2 Basic vector (Promega) as described in (Feng, Lawson et al. 2008). FSHβ promoter was created by amplifying -1990 to +1 bp of FSHβ promoter gene from murine genomic DNA and inserted into pGL2 vector.

3.5.3 Construction of siRNA constructs

3.5.3.1 Design of oligonucleotides

The oligonucleotides for ACTN4 short hairpin RNA (shRNA) construct were designed based on the mRNA sequence. The target region for knocking down was determined by BLOCK-iT™ RNAi Designer (Invitrogen). Two pairs of oligos were synthesized to target ACTN4 as shown in Table 3.9. The underlined sequences indicate the shRNA targeting regions.

Table 3.9 Oligonucleotides designed for synthesis of ACTN4 short hairpin RNA.

Name	Sequence (5'-3') Forward Sequence (5'-3') Reverse
siACTN4$_{976}$	GATCCCCGCACCTGATGGAAGACTATTTCAAGAGA ATAGTCTTCCATCAGGTGCTTTTTA
	AGCTTAAAAAGCACCTGATGGAAGACTATTCTCTTGAAAT AGTCTTCCATCAGGTGCGGG
siACTN4$_{2032}$	GATCCCCGCAATCCAATGAGCACCTTTTCAAGAGA AAGGTGCTCATTGGATTGCTTTTTA
	AGCTTAAAAAGCAATCCAATGAGCACCTTTCTCTTGAAAT AAGGTGCTCATTGGATTGCGGG

3.5.3.2 Annealing of oligonuleotides

The shRNA oligonucleotides were dissolved and diluted to a concentration of 3 µg/µL in nuclease-free H_2O and the annealing reaction was set up by mixing 1 µL of each oligo with 48 µL annealing buffer (100 mM NaCl and 50 mM HEPES, pH 7.4). The annealing mixture was first heated to 90 °C for 4 min, and then was switched to 70 °C for 10 min, after which the reaction mixture was cooled down to 37 °C for 20 min, and finally was kept at 4 °C for 2 h.

3.5.3.3 Restriction digestion of pSUPER vector

The pSUPER Basic vector (Oligoengine) (1 µg) was linearized with HindIII and BglII. Digestion was performed at 37 °C. Enzymes were then inactivated at 65 °C for 15 min. The linearized vector was loaded on a 1.1 % agarose gel and purified using the Gel purification kit (Qiagen).

3.5.3.4 Ligation of annealed oligos and linearized vector

Ligation mixture was assembled as shown in Table 3.10, and the reaction was carried out at RT for 2 h. After transformation, clones were selected for sequencing to confirm the shRNA construct.

Table 3.10 ligation of annealed shRNA oligo with pSUPER vector.

Components	Volume (μL)
Annealed oligonucleotides	1
Linearized pSUPER vector (0.3 μg/uL)	1
10X T4 DNA ligase buffer (Promega)	1
T4 DNA ligase (3 U/μL, Promega)	1
Nuclease-Free Water	6
Total volume	10

3.5.4 Plasmid isolation and verification

Plasmid DNA of selected colonies was isolated using Wizard Plus Miniprep Kit (Promega). The insert sequence of each construct was verified by DNA sequencing. Sequencing PCR was assembled and cycling parameters were listed in Table 3.11, using corresponding sequencing primers indicated by vector map. After sequencing PCR, the DNA was precipitated with 20 μL of 3 M NaOAc (pH 4.6) and 50 μL 95 % ethanol for 30min at -20 °C, followed by centrifugation at 13,000 rpm for 30 min at 4 °C. The DNA pellet was washed twice with 70 % ethanol and dried before sending out for sequencing.

Table 3.11 Sequencing PCR mixture and cycling parameters.

Components	Volume (μL)
DNA template	250-500 ng
5X sequencing buffer (Applied Biosystems)	2
Primer (5 μM)	1
Bigdye Version 3 (Applied Biosystems)	1
Nuclease-Free Water to final volume	10

Step	Cycle	Temperature (°C)	Time
1	25	96 50 60	30s 15s 4 min
2	1	4	∞

3.6 Luciferase promoter analysis

Cells were seeded in 24-well plates. After overnight plating, 500 ng/well of over-expression/shRNA plasmids, 500 ng/well of the luciferase reporter plasmids and 25 ng/well of simian virus 40 (SV40) were transient transfected into the cells and the cells were continued to culture for 48-72h. Cells were then rinsed with ice-cold PBS, and the luciferase activity was measured by using the Dual-Glo system (Promega) after splitting the cell lysate into 96well-white plate, in a Verita Microplate luminometer (Turner Biosystem). Luciferase activity was normalized to the levels of *Renilla* luciferase. The results were expressed as the fold change over basal levels, which were the values that were generated from transfection of the luciferase reporter plasmids with empty vector/shGFP. Statistical analysis was done by paired *t*-test.

3.7 Western blot

The protein sample preparation and quantitation were performed as described in 3.2. For each sample, 25 μg of protein was mixed with 2× SDS loading buffer and heated for 10 min at 95 °C before loading on a 10-12 % SDS polyacrylamide gel (PAGE). Constant voltage at 80 V was set for the first 20 min, and followed by increasing to constant voltage at 130 V.

The proteins were then transferred to an Immuno-Blot PVDF (Bio-Rad) membrane in the transfer buffer at a constant voltage of 80 V for 1 h at 4 °C. Once the transfer was completed, the membrane was taken out and incubated with a series of buffers (Table 3.12). All the incubation and wash steps were performed on a rotating platform. The membrane was first blocked with the blocking buffer for 1 h at RT, followed by washing

three times in the washing buffer. And then it was incubated with the primary antibodies (Table 3.13) diluted in the antibody dilution buffer for 1 h at RT, or overnight at 4 ^{o}C. Subsequently, washing was carried out as described above. The membrane was then incubated with the appropriate secondary antibody diluted in the antibody dilution buffer for 1 h at RT. The membrane was then washed as described above and then incubated with the SuperSignal® WestPico Chemiluminescent substrate (Pierce) for 5 min. After rinsing in H_2O for 5 sec, the membrane was placed in the middle of two transparent plastic sheets. Autoradiography was performed using X-ray film (Kodak Scientific Imaging Film X-OMAT^{TM}) in an autoradiograph cassette. Exposure time was optimized from 10 sec until the demanded band was detected. The film was developed using a Kodak M35 X-OMAT Processor.

Table 3.12 Buffers used in western blot.

Buffers	Component
2 X loading buffer	100 mM Tris-HCl pH 6.8, 200 mM β-ME, 4 % SDS, 0.2 % Bromophenol blue, 20 % glycerol
Running buffer	25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1 % SDS
Transfer buffer	25 mM Tris-HCl pH 8.3, 192 mM glycine
Washing buffer (TBST)	10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 % Tween-20
Blocking buffer	TBST with 10 % non-fat milk
Antibody dilution buffer	TBST with 1 % non-fat milk

Table 3.13 Antibodies used in western blot and confocal microscopy.

Antibodies	Source	Dilution (WB)	Dilution (confocal)
ACTN 4	Santacruz Biotechnology	1: 1000	1:100
Actin	BD Transduction Laboratories	1: 5000	NA
NPM	Sigma	1: 5000	NA
FLAG	Sigma	1: 2000	NA
Cacybp	Santacruz Biotechnology	1: 5000	NA
β-catenin	Abcam	1: 1000	1:200
GAPDH	Santacruz Biotechnology	1: 3000	NA
GSK3β	Santacruz Biotechnology	1:2000	NA
p-GSK3 β	Santacruz Biotechnology	1:2000	NA
Sf-1	Upstate	1:2000	NA
Goat anti rabbit IgG HRP	Santacruz Biotechnology	1:5000	NA
Bovine anti goat IgG HRP	Santacruz Biotechnology	1:5000	NA
Rabbit anti mouse IgG HRP	Santacruz Biotechnology	1:5000	NA
Alexa Fluor-546 conjugated donkey-anti goat IgG	Invitrogen	NA	1:200
Alexa Fluor-594 conjugated goat-anti rabbit IgG	Invitrogen	1:200	NA

3.8 Confocal microscopy

Coverslips (VWR) were pre-cleaned and kept in 70 % ethanol. Just before coating, they were rinsed in 100 % ethanol, and dried. The coverslips were then sterilized by Ultraviolet (UV), and 0.1 % poly-L-Lysine (Sigma) solution was applied at 300 µL/coverslip. After incubation for 30 min, the solution was removed and the coverslips were dried and further sterilized with UV for 30 min. LβT2 cells were seeded on poly-L-lysine (Sigma) coated sterilized coverslips in 6-well plates and starved in serum free medium overnight before treatment with GnRH (Sigma). After treated for the indicated times, cells were washed twice with ice-cold DPBS (PBS with Ca^{2+} and Mg^{2+}), and then fixed in 3 % paraformaldehyde (PFA) (Sigma) at RT for 15 min followed by washing three times with 50 mM NH_4Cl in DPBS. They were rinsed in DPBS and permeabilized with DPBS containing 0.5 % saponin (Sigma) at RT for 15min. After permeabilization, the fixed cells were blocked in DPBS containing horse serum (10 %) plus BSA (1 %) at RT for 1 h. Incubation with primary antibodies (Table 3.13) was carried out for 1 h at RT or overnight at 4 °C. The cells were washed 3 × 3 min in DPBS containing 0.1 % saponin, and incubated with the secondary antibodies (Table 3.13) for 1 h at RT, followed by 6 × 2 min washing in DPBS containing 0.1 % saponin. The stained cells were then mounted with DAPI (4'6-diamidino-2-phenylindole)-contained mounting solution (Santacruz), and examined by a LSM510 confocal fluorescence microscope (Carl Zeiss). For fluorescence quantitation assays, the unit fluorescence in the nucleus (I_n) and the whole cell (I_w) were calculated as intensity/area. The results were shown as the fold change over mock-treated controls. A minimum of 50 cells (i.e., ±10 microscopic fields) in total were counted. Statistical analyses were performed by two-sample homoscedastic *t*-test.

3.9 Promoter pull-down assay

3.9.1 Generating the mutated LHβ promoters

The intact rat LHβ (rLHβ) promoter in CAT6 vector is a general gift given by A/P Melamed. There are two putative Sf-1 binding sites, which are located at the proximal region -59 bp and distal region -119 bp of the intact rLHβ promoter, respectively (Melamed, Kadir et al. 2006). The 3 mutated rLHβ promoters, which have no Sf-1 binding sites or only one Sf-1 binding site, were generated by QuickChange™ site-directed mutagenesis kit (Stratagene) following the instruction using each pair of primers (Table 3.14).

Table 3.14 The primers used for generating mutated rLHβ promoters.

Construct Name	Template	Primers	Sequence (5'-3') Forward Sequence (5'-3') Reverse
rLHβ ΔSf-1_proximal	rLHβ-CAT6	rLHβΔSf-1_p_F	GCCTCTGCTTAGTGGAATTCCCA CCCCCACAACCCGCAGG
		rLHβΔSf-1_p_R	CCTGCGGGTTGTGGGGGTGGGA ATTCC ACTAAGCAGAGGC
rLHβ ΔSf-1_distal	rLHβ-CAT6	rLHβΔSf-1_d_F	GCTGGTCCCTGGCTTTTCTGAAA TTGTCTGTCTCGCCCCCAAAG
		rLHβΔSf-1_d_R	CTTTGGGGGCGAGACAGACAAT TTCAGAAAAGCCAGGGACCAGC
rLHβ ΔSf-1_p&d	rLHβ ΔSf-1_distal	rLHβΔSf-1_p_F	GCCTCTGCTTAGTGGAATTCCCA CCCCCACAACCCGCAGG
		rLHβΔSf-1_p_R	CCTGCGGGTTGTGGGGGTGGGA ATTCC ACTAAGCAGAGGC

(Mutated nucleotides were underlined.)

3.9.2 Generating the biotin-labeled intact and mutated LHβ promoters

The biotin-labeled promoters were generated by PCR amplification. In each PCR reaction, each of the promoters generated in 3.9.1 was used as a template, and biotin-labeled promoter was amplified using 5'-biotin-labeled forward primer (5'-GCTGGTCCCTGGCTTTTCTG-3'), which starts from the -145 bp relative to the transcription start site 0 bp, and the normal reverse primer (5'-ACCTTCCCTACCTTGGGCAC-3'), which starts from the transcription start site; the control non-biotin-labeled promoters was amplified using the same forward primer but without 5' biotin-labeling, and the normal reverse primer. The PCR reaction was carried out in a 50 µL reaction, with 100 ng of each template, the composition of the reaction was previously shown in Table 3.8. The mixture was first denatured at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 sec, 59 °C for 1 min, and 72 °C for 20 sec, and finally extended at 72 °C for 5 min.

3.9.3 Promoter pull-down assay

For each reaction, 500 µg of nuclear extract was mixed with 50 µL of the biotin-labeled promoter alone or together with competitive non-biotin-labeled promoter, and incubated overnight at 4 °C with gentle rotation. On the next day, 20 µL of streptavidin beads was then added and incubated with the mixture for another 4 h at 4 °C with gentle rotation. The protein-promoter-bead complex was then washed 3 times in TBST buffer and was eluted by 2× reduced SDS loading buffer and applied to western blot analysis. The non-biotin-labeled promoter alone mixed with nuclear extraction was used as a binding control.

Chapter 4. ICAT proteomic profiling of the protein responses induced by GnRH in gonadotrope cell line

4.1 Introduction

GnRH is the central regulator of the reproductive system through stimulating the synthesis and secretion of gonadotropins LH and FSH from pituitary (Tsai 2006). GnRH activates multiple downstream signaling through binding to the type I GnRH receptor, and subsequently transmitting the signals via the heterotrimeric Gq protein and inducing the elevation of intracellular Ca^{2+} level, activation of PKC/MAPK signaling cascades, which then promoting gonadotropins transcription and secretion (Reiss, Llevi et al. 1997). GnRH agonist (GnRHa) analogs have been used to treat steroid-dependent diseases (Schultze-Mosgau, Griesinger et al. 2005) and proved to be a safe and efficient class of drugs. As the important clinical applications of GnRHa, the signal transduction pathways of GnRH, therefore, are of great interests to be identified. However, MAPK cascades are the only well studied and characterized pathways so far (Reiss, Llevi et al. 1997; Harris, Bonfil et al. 2002; Harris, Chuderland et al. 2003; Bonfil, Chuderland et al. 2004; Naor 2009). Recent studies suggested Wnt signaling may also involve in mediating GnRH response (Gardner, Maudsley et al. 2007; Salisbury, Binder et al. 2008; Gardner and Pawson 2009; Gardner, Stavrou et al. 2010), and most findings were focused on β-catenin, which is known to be the key effecter of canonical Wnt signalling.

It is well known that in the Wnt/canonical pathway β-catenin is activated by Wnt ligand through acting on a destruction complex, composed of casein kinase I (CKI) and glycogen synthase kinase-3β (GSK-3β) (Huelsken and Behrens 2002). The activated β-catenin then functions as a coactivator with the transcription factors T-cell factor (TCF)/ lymphoid enhancer factor (LEF) in mediating the transcription of Wnt target genes such as *c-Myc*, *c-Jun*, *Fra-1*, and others (Moon, Kohn et al. 2004; Nelson and Nusse 2004).

Recent studies indicated that β-catenin and the TCF target gene *c-Jun* are likely involved in mediating GnRH induced LHβ gene transcription (Salisbury, Binder et al. 2007; Salisbury, Binder et al. 2009). Although these results highlighted the possibility of crosstalk between Wnt signalling and GnRH signalling in the field of gonadotrope biology, the knowledge however is only limited to a few mediators, which is hard for the elucidation of a full spectrum of the signalling pathway.

Previous signaling pathway studies in gonadotrope were mostly performed at the transcriptomic level, such as extensive microarray studies of 1 to 6 h of GnRH treatment time course done by Sealfon's group (Wurmbach, Yuen et al. 2001; Ruf, Fink et al. 2003; Ruf and Sealfon 2004), a similar microarray study of 1 h and 24 h of GnRH treatment done by Shawn's group (Kakar, Winters et al. 2003), and a microarray analysis of GnRH pulse frequency treatment done by Lawson's group (Lawson, Tsutsumi et al. 2007). The results obtained from these transcriptomic studies turned out to advance the understanding of GnRH-induced MAPK pathways. However, so far no high-throughput proteomics study has been carried out to study the GnRH signaling. In order to address the research gap, we carried out cICAT-based proteomics experiment to identify differentially expressed proteins between mock-treated and GnRH-treated LβT2 cells. cICAT labeling, which only targets cysteine residues (Gygi, Rist et al. 1999; Hansen, Schmitt-Ulms et al. 2003; Li, Steen et al. 2003), greatly simplifies the MS of peptide mixture and may thereby facilitating the identification of low abundant proteins such as signaling molecules.

Our study identified a number of gene products regulated by GnRH while involve in post translation modifications (PTMs), furthermore we found some of these proteins are

potential mediators of GnRH response through Wnt/ β-catenin signaling. On the basis of our data, we proposed novel signaling pathways, which advance the knowledge of crosstalk between Wnt signaling and GnRH signaling in the field of gonadotrope biology.

4.2 Results

4.2.1 cICAT proteomic profiling of whole cell lysate from mock-treated and GnRH-treated LβT2 cells.

Total protein expression profiles of 4 h mock-treated and GnRH-treated LβT2 cells were analyzed by cICAT-based proteomics experiments. Three independent experiments were conducted as biological replicates. After treatment, total proteins were isolated from individual samples and subjected to cICAT labeling. The details of the sample preparation were described in chapter 3, and the flow chart of cICAT experiments is shown in Figure 4.1.

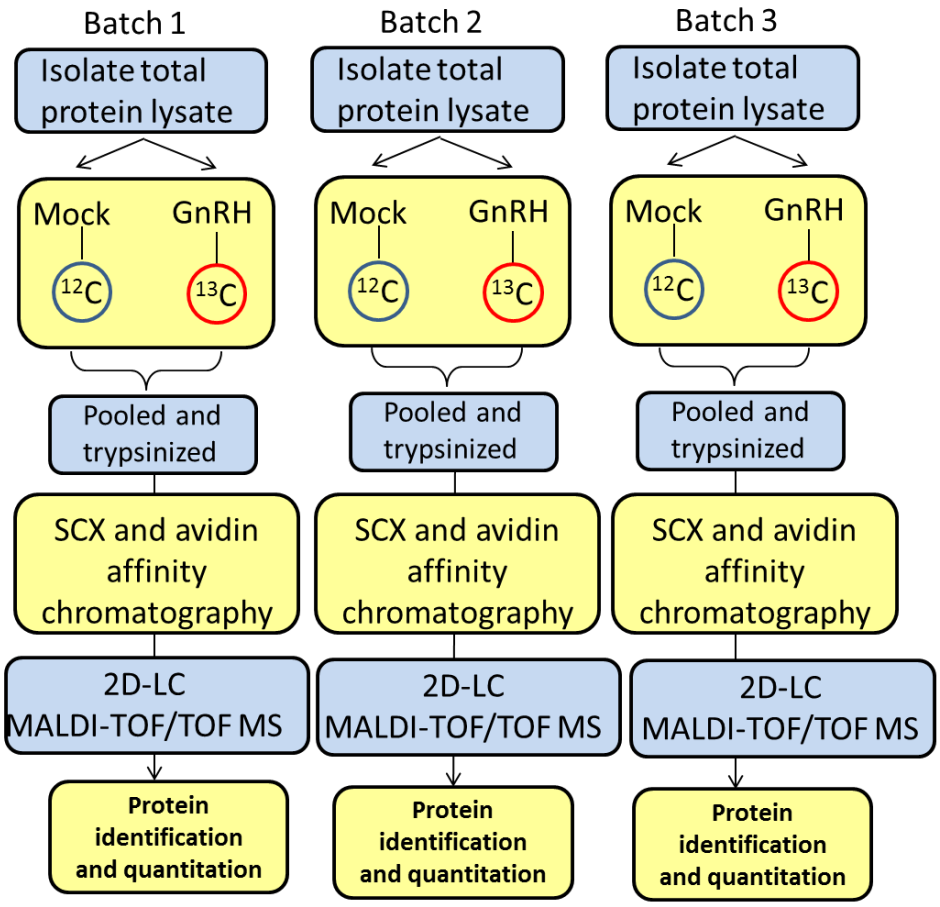


Figure 4.1 Schematic representation of the workflow of the cICAT–based proteomic profiling

cICAT labeling were carried out in triplicates of whole cell lysate from mock-treated and GnRH-treated LβT2 cells**.** Mock-treated total protein extracts were labeled with light cICAT reagent-^{12}C, and those from GnRH-treated cells were labeled with heavy cICAT reagent-^{13}C.

The minimum ion score C.I. % was set as the value to which no more than 5% FDR was achieved. Based on this cut-off threshold, all the proteins identified from the random database search were single-peptide matches. With this filter, a total of 364 proteins from triplicate 1, 866 proteins from triplicate 2, and 797 proteins from triplicate 3 were confidently identified by matching MS/MS data to the protein sequences by MASCOT database search. A total of 307 unique proteins were commonly identified from at least two of the three independent experiments (Supplementary soft copy). The cellular distributions of the 307 proteins were analyzed by Ingenuity Pathway Analysis server (Figure 4.2). The result showed that about 26 % of proteins were from nucleus, 22 % of proteins were from cytoplasm, 14 % of proteins were from plasma membrane, 13 % of proteins were from mitochondria, 10 % of proteins were from cytoskeleton, 9 % of proteins were from extracellular, 4 % of proteins were from ribosome and 2% of proteins remained unknown.

The ratios of differentially expressed proteins between mock-treated and GnRH-treated cells were calculated by GPS Explorer^{TM} software (Ver. 3.6). Each ratio was obtained by normalizing against median ratio of all the cICAT peptides detected in one sample. The ratios were then calculated by comparison of the clustered heavy-cICAT labeled peptides with light-cICAT labeled peptides, and the cut-off was fixed at 1.3 for up-regulation and 0.77 for down-regulation. Applying these cut-off ratios to the three sets of biological triplicates, those common proteins which meet the criteria in at least two sets of the biological triplicates were selected as the significantly altered proteins. Here we identified 194 proteins as significantly altered proteins. As most of these proteins (93.8 %) were present twice in three experiments, the correlation of the protein ratios in two

datasets was plotted. For those proteins present in all three experiments, only the two most closed ratios were selected for correlation study (Figure 4.3).

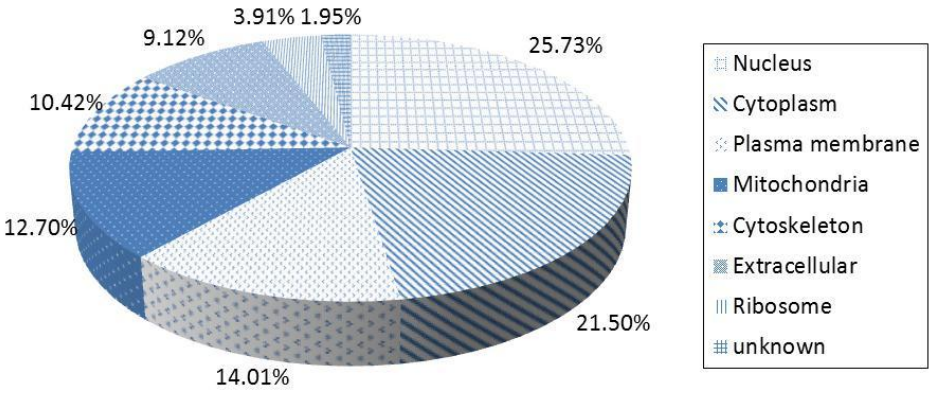


Figure 4.2 Cellular distribution of all the unique proteins identified by cICAT proteomics profiling.

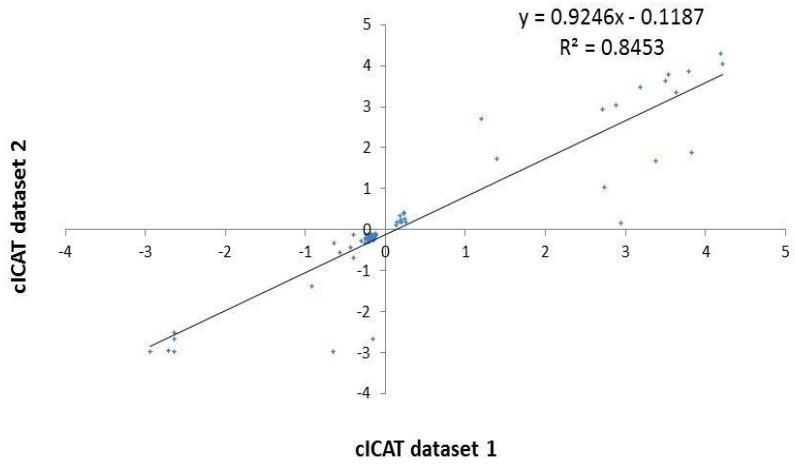


Figure 4.3 Correlation between the cICAT ratios of the significantly altered proteins by GnRH-treatment from two independent datasets.

The correlation of the cICAT ratios of the 194 significantly altered proteins from 2 datasets were determined by using the Log10 value of each ratio. The correlation coefficiency (R^2) was shown as 0.845, indicating the acceptable variations in both datasets.

4.2.2 Gene Oncology studies of the significantly altered proteins

Gene ontology studies using DAVID were carried out for the significantly altered proteins by GnRH-induction. GO distributions in molecular function were classified and demonstrated in Figure 4.4. Surprisingly, phosphoprotein and acetylation were found as the top 2-ranked functions comprising the most number of significantly altered proteins induced by GnRH. These two functions are closely correlated to our research topic--regulation of gonadotropins. The corresponding proteins list involved in phosphorylation were shown in Table 4.1, and the proteins involved in acetylation were shown in Table 4.2.

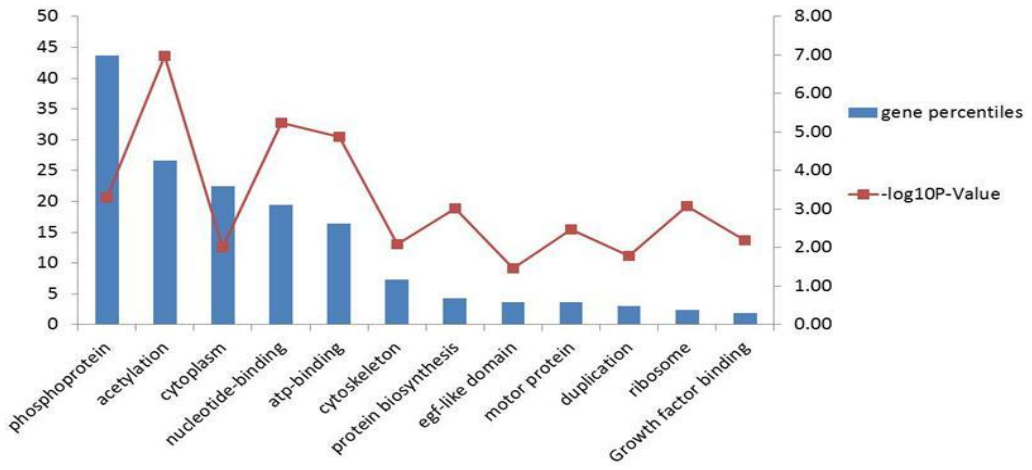


Figure 4.4 GO analysis of significantly regulated proteins by GnRH-induction.

GO analysis were done using DAVID software, the percentiles of genes involved in each molecular function were plotted against the corresponding molecular function (bar). The -Log_{10} P-value of each molecular function was also indicated in the graph (line). Only the molecular functions with $P < 0.05$ were shown in this graph.

Table 4.1 Significantly altered proteins which are involve in phosphorylation.

(↑ refers to up-regulation; ↓ refers to down-regulation; Sp1, Sp2, Sp3 refers to each of the 3 independent experiments)

Accession Number	Protein Name	Avg ICAT Ratio (H/L)			Exp Pat.
		Sp1	Sp2	Sp3	
IPI00136929	Actg1 Gamma actin-like protein	879.14		1.46	↑
IPI00648173	Cltc Clathrin, heavy polypeptide	1.38		1.56	↑
IPI00323064	Pdcd4 Programmed cell death protein 4	6101.59	7184.27		↑
IPI00667859	Gm6438 similar to Ribosomal protein S27a		1512.09	2906.32	↑
IPI00828704	Btk Bruton agammaglobulinemia tyrosine kinase		767.39	1083.46	↑
IPI00830276	Adcy10 Isoform 2 of Adenylate cyclase type 10	2414.80	46.86		↑
IPI00757256	Myo16 Isoform 2 of Myosin-XVI		15.89	517.92	↑
IPI00459500	Obscn obscurin isoform 1	554.06	10.90		↑
IPI00650012	Dnajc7 DnaJ (Hsp40) homolog, subfamily C, member 7 (Fragment)		1.78	1.51	↑
IPI00230044	Tpm3 Isoform 2 of Tropomyosin alpha-3 chain		1.71	2.54	↑
IPI00405364	Ddx17 Isoform 2 of Probable ATP-dependent RNA helicase DDX17		1.60	1.58	↑
IPI00224109	Hsph1 Isoform HSP105-beta of Heat shock protein 105 kDa		1.54	2.26	↑
IPI00658779	Gm6563 hypothetical protein isoform 2		1.50	1.50	↑
IPI00663627	Flnb Filamin-B	20.17	1.35	1.27	↑
IPI00115650	Cacybp Calcyclin-binding protein	0.67	0.82	0.65	↓
IPI00621229	Rps8-ps1 Ribosomal protein S8	0.74	0.81	0.76	↓

IPI00123494	Psmd2 26S proteasome non-ATPase regulatory subunit 2	0.71	0.79	0.57	↓
IPI00117016	Mcm4 DNA replication licensing factor MCM4		0.76	0.76	↓
IPI00130173	Uba2 SUMO-activating enzyme subunit 2	0.00	0.75	0.79	↓
IPI00874963	Gpbp1l1 Vasculin-like protein 1	0.57	0.74		↓
IPI00112555	Gars Glycyl-tRNA synthetase	0.66	0.73	0.60	↓
IPI00886245	Gdi1 Guanosine diphosphate (GDP) dissociation inhibitor 1		0.73	0.64	↓
IPI00116310	Dnahc2 Putative uncharacterized protein Dnahc2	0.67	0.71		↓
IPI00653598	Uqcrc1 cytochrome b-c1 complex subunit 1, mitochondrial precursor	0.67	0.70	0.63	↓
IPI00130491	Prkch Protein kinase C eta type		0.69	0.56	↓
IPI00321446	Prkca Protein kinase C alpha type		0.69	0.60	↓
IPI00321647	Eif3c Eukaryotic translation initiation factor 3 subunit C		0.68	0.58	↓
IPI00126898	Scn11a Sodium channel protein type 11 subunit alpha		0.66	0.00	↓
IPI00125091	Lasp1 LIM and SH3 domain protein 1		0.65	0.67	↓
IPI00223757	Akr1b3 Aldose reductase		0.65	0.67	↓
IPI00649191	Psme3 Proteaseome (Prosome, macropain) 28 subunit, 3		0.64	0.68	↓
IPI00849793	Rpl12 60S ribosomal protein L12	0.40	0.53	0.21	↓
IPI00399702	Phf6 Isoform 2 of PHD finger protein 6	0.71	0.45	0.74	↓
IPI00131376	Spnb1 spectrin beta chain, erythrocyte		0.23	0.47	↓
IPI00409393	Ltbp1 Putative uncharacterized protein Ltbp1	0.00	0.00	0.00	↓

IPI00349296	Tanc1 Isoform 1 of Protein TANC1		0.00	0.00	↓
IPI00122486	Camkv CaM kinase-like vesicle-associated protein		0.00	0.00	↓
IPI00378681	Ubr4 Isoform 1 of E3 ubiquitin-protein ligase UBR4		0.00	0.00	↓
IPI00608114	Mycbp2 probable E3 ubiquitin-protein ligase MYCBP2	0.00	0.00	0.00	↓
IPI00648094	Tk1 Thymidine kinase, cytosolic	0.00	0.00	0.00	↓
IPI00857004	Chd4 50 kDa protein	0.00	0.00	0.00	↓
IPI00944011	Ripk3 receptor-interacting serine/threonine-protein kinase 3 isoform 2	0.00	0.00	0.00	↓
IPI00127176	6720456B07Rik Probable protein BRICK1		0.00	0.00	↓
IPI00127449	Zfp622 Zinc finger protein 622		0.00	0.00	↓
IPI00130218	Kif11 Kinesin-like protein KIF11		0.00	0.00	↓
IPI00230680	Atp4a Potassium-transporting ATPase alpha chain 1		0.00	0.00	↓
IPI00408595	Wnk2 Isoform 2 of Serine/threonine-protein kinase WNK2		0.00	0.00	↓
IPI00421063	Brca1 breast cancer type 1 susceptibility protein homolog		0.00	0.00	↓
IPI00649450	Gstm1 23 kDa protein		0.00	0.00	↓
IPI00653178	1190007F08Rik Putative uncharacterized protein		0.00	0.00	↓
IPI00666451	Trpm1 Isoform 2 of Transient receptor potential cation channel subfamily M		0.00	0.00	↓
IPI00762214	Trrap 434 kDa protein		0.00	0.00	↓
IPI00556723	Dnm1l Isoform 1 of Dynamin-1-like protein		0.00	0.43	↓
IPI00119876	Dync1h1 Cytoplasmic dynein 1 heavy chain 1		0.00	0.57	↓

IPI00272622	Metap1 Methionine aminopeptidase 1	0.00	0.00		↓
IPI00605122	Btbd7 Isoform 3 of BTB/POZ domain-containing protein 7	0.00	0.00		↓
IPI00113348	Fam59a Isoform 1 of Protein FAM59A	0.00		0.00	↓
IPI00124178	Sptlc2 Serine palmitoyltransferase 2	0.00		0.00	↓
IPI00229217	Mapk15 Mitogen-activated protein kinase 15	0.00		0.00	↓
IPI00308198	Ubxn2b UBX domain-containing protein 2B	0.00		0.00	↓
IPI00347275	Vps24 Charged multivesicular body protein 3	0.00		0.00	↓
IPI00410869	Lrp8 Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	0.00		0.00	↓
IPI00460692	4933407H18Rik Putative uncharacterized protein 4933407H18Rik	0.00		0.00	↓
IPI00480458	Itgb4 Isoform 3 of Integrin beta-4	0.00		0.00	↓
IPI00605176	Trio Isoform 2 of Triple functional domain protein	0.00		0.00	↓
IPI00649093	Myo18a Myosin XVIIIa	0.00		0.00	↓
IPI00742341	Nalcn sodium leak channel non-selective protein	0.00		0.00	↓
IPI00918828	Plxna2 Protein	0.00		0.00	↓
IPI00850539	LOC100044138 similar to CDCrel-1AI isoform 1	0.50		0.54	↓
IPI00420835	Itga6 Isoform Alpha-6X1B of Integrin alpha-6	0.00		0.61	↓
IPI00828969	Pdlim5 Isoform 1 of PDZ and LIM domain protein 5	0.00		0.76	↓
IPI00120374	Ppp2ca Serine/threonine-protein phosphatase 2A catalytic subunit alpha iso	0.40		0.77	↓

Table 4.2 Significantly altered proteins which are involve in acetylation.

(↑ refers to up-regulation; ↓ refers to down-regulation; Sp1, Sp2, Sp3 refers to each of the 3 independent experiments)

Accession Number	Protein Name	Avg ICAT Ratio (H/L)			Exp Pat.
		Sp1	Sp2	Sp3	
IPI00875110	Idh2 52 kDa protein		16421.24	10936.26	↑
IPI00667859	Gm6438 similar to Ribosomal protein S27a		1512.09	2906.32	↑
IPI00828704	Btk Bruton agammaglobulinemia tyrosine kinase		767.39	1083.46	↑
IPI00230044	Tpm3 Isoform 2 of Tropomyosin alpha-3 chain		1.71	2.54	↑
IPI00648173	Cltc Clathrin, heavy polypeptide	1.38		1.56	↑
IPI00650012	Dnajc7 DnaJ (Hsp40) homolog, subfamily C, member 7 (Fragment)		1.78	1.51	↑
IPI00658779	Gm6563 hypothetical protein isoform 2		1.50	1.50	↑
IPI00136929	Actg1 Gamma actin-like protein	879.14		1.46	↑
IPI00663627	Flnb Filamin-B	20.17	1.35	1.27	↑
IPI00885495	Cmc1 COX assembly mitochondrial protein homolog	6639.63	76.47		↑
IPI00130173	Uba2 SUMO-activating enzyme subunit 2	0.00	0.75	0.79	↓
IPI00120374	Ppp2ca Serine/threonine-protein phosphatase 2A catalytic subunit alpha iso	0.40		0.77	↓
IPI00828969	Pdlim5 Isoform 1 of PDZ and LIM domain protein 5	0.00		0.76	↓
IPI00117016	Mcm4 DNA replication licensing factor MCM4		0.76	0.76	↓
IPI00403706	Rnpep aminopeptidase B isoform 2		0.76	0.72	↓

IPI00309398	Mcm5 DNA replication licensing factor MCM5		0.71	0.71	↓
IPI00128699	Snrpn Small nuclear ribonucleoprotein-associated protein N	0.57	0.62	0.70	↓
IPI00649191	Psme3 Proteaseome (Prosome, macropain) 28 subunit, 3		0.64	0.68	↓
IPI00125091	Lasp1 LIM and SH3 domain protein 1		0.65	0.67	↓
IPI00223757	Akr1b3 Aldose reductase		0.65	0.67	↓
IPI00115650	Cacybp Calcyclin-binding protein	0.67	0.82	0.65	↓
IPI00310220	Gnb4 Guanine nucleotide-binding protein subunit beta-4	0.62	0.77	0.64	↓
IPI00653598	Uqcrc1 cytochrome b-c1 complex subunit 1, mitochondrial precursor	0.67	0.70	0.63	↓
IPI00321446	Prkca Protein kinase C alpha type		0.69	0.60	↓
IPI00112555	Gars Glycyl-tRNA synthetase	0.66	0.73	0.60	↓
IPI00321647	Eif3c Eukaryotic translation initiation factor 3 subunit C		0.68	0.58	↓
IPI00649986	Psme2 proteasome activator complex subunit 2 isoform 2	0.42	0.56	0.58	↓
IPI00123494	Psmd2 26S proteasome non-ATPase regulatory subunit 2	0.71	0.79	0.57	↓
IPI00119876	Dync1h1 Cytoplasmic dynein 1 heavy chain 1		0.00	0.57	↓
IPI00649328	Cisd3 Cisd3 protein	0.72	0.84	0.55	↓
IPI00113849	Cdc42 Isoform 2 of Cell division control protein 42 homolog		0.64	0.53	↓
IPI00556723	Dnm1l Isoform 1 of Dynamin-1-like protein		0.00	0.43	↓
IPI00849793	Rpl12 60S ribosomal protein L12	0.40	0.53	0.21	↓

IPI00378681	Ubr4 Isoform 1 of E3 ubiquitin-protein ligase UBR4		0.00	0.00	↓
IPI00453769	Cops7b COP9 signalosome complex subunit 7b		0.00	0.00	↓
IPI00127176	6720456B07Rik Probable protein BRICK1		0.00	0.00	↓
IPI00762214	Trrap 434 kDa protein		0.00	0.00	↓
IPI00857004	Chd4 50 kDa protein	0.00	0.00	0.00	↓
IPI00608114	Mycbp2 probable E3 ubiquitin-protein ligase MYCBP2	0.00	0.00	0.00	↓
IPI00130218	Kif11 Kinesin-like protein KIF11		0.00	0.00	↓
IPI00136012	Ggcx Vitamin K-dependent gamma-carboxylase	0.00	0.00	0.00	↓
IPI00409255	D030016E14Rik Isoform 1 of UPF0636 protein C4orf41 homolog	0.00		0.00	↓
IPI00318464	Eif1b Eukaryotic translation initiation factor 1b		0.00	0.00	↓
IPI00272622	Metap1 Methionine aminopeptidase 1	0.00	0.00		↓

Protein phosphorylation is one of the PTMs which is caused by the changing in metabolic activity, environmental conditions and hormonal signals (Karin 1994). Phosphorylation of transcription factors (TFs) modulate their transactivation activities through affecting the DNA-binding activity, the transcriptional activity and the subcellular localization of the TFs (Hunter and Karin 1992). In addition, phosphorylation has been found to be required for the signal transduction from cell surface to the nucleus in many cell contexts as well as GnRH-induced MAPK cascades in gonadotrope cells, reviewed by (Naor 2009). Acetylation is another crucial PTM process that plays a key role in regulating gene expression. It is widely presented in chromatin proteins (Choudhary, Kumar et al. 2009). Except for regulating histone, acetylation is also implicated in the regulation of transcription factors, effecter proteins, molecular chaperons, and cytoskeleton proteins. Recent study even suggested that acetylation may crosstalk with other PTMs, including phosphorylation, methylation, ubiquitination, sumoylation for dynamic control of cellular signaling (Yang and Seto 2008). The studies in gonadotrope cell line also indicated that GnRH may regulate gonadotropin through modulating acetylation process, reviewed by (Melamed, Kadir et al. 2006). As both phosphorylation and acetylation are likely involved in regulating GnRH signaling, we therefore continue to investigate the potential pathways which may be modulated by these two regulatory processes. We utilized STRING software to analyze the protein-protein networks, which may help to discover the underlying mechanism. Under the "action mode", we found both Cacybp and Ddx17 act on β-catenin. As β-catenin is a key effecter of the Wnt signaling, and GnRH was recently found to target Wnt signaling, we further looked for other potential factors which

may be involved in Wnt signaling through literature mining. Finally, we identified a group of proteins which may be potentially involved in this process (Figure 4.5).

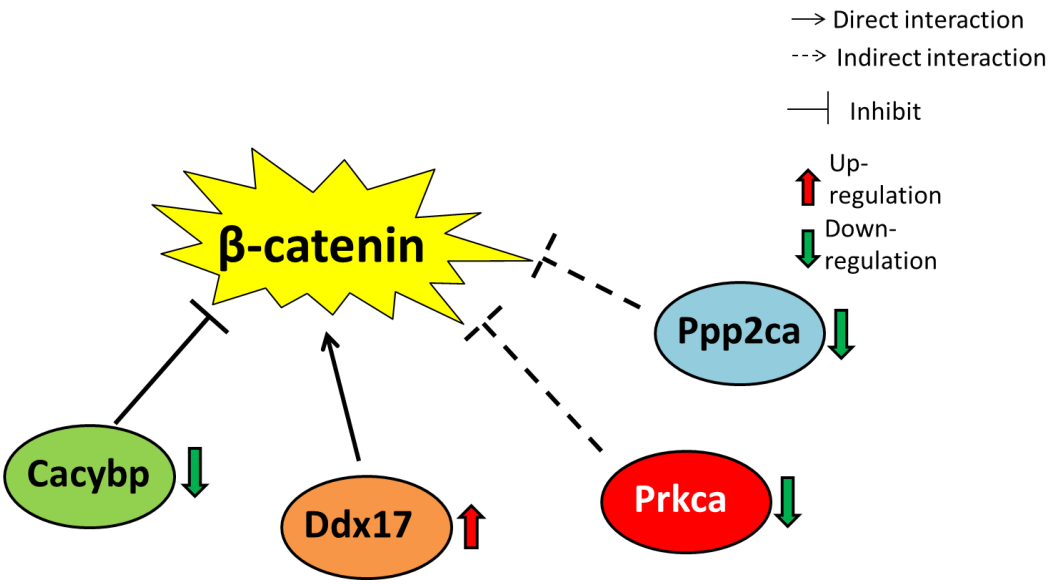


Figure 4.5 Predicted Wnt signaling players among the significantly altered proteins.

The actions of Cacybp and Ddx17 on β-catenin were identified by STRING under "action mode". Ppp2ca and Prkca were identified by literature mining.

Ppp2ca was down-regulated by GnRH treatment. Ppp2ca is the catalytic subunit of PP2A kinase (Janssens and Goris 2001), which was suggested to be essential for both GSK-3β-dependent (Ivaska, Nissinen et al. 2002; Hildesheim, Belova et al. 2003; Lee, Seo et al. 2005; Mitra, Menezes et al. 2012), and GSK-3β-independent degradation of β-catenin (Zhang, Yang et al. 2009). Ddx17 was found to be up-regulated by GnRH treatment. Ddx17 was previously found to act as a coactivator of β-catenin through forming a complex with β-catenin in the nucleus and subsequently promoting the β-catenin target gene expression (Shin, Rossow et al. 2007). Cacybp was found to be down-regulated by GnRH. It is known to be one of the component of E3 ligase complex which regulates the degradation of β-catenin through GSK-3β independent mechanisms (Filipek 2006). Recently, Cacybp was also identified as a novel phosphatase which modulates ERK 1/2 activity (Kilanczyk, Filipek et al. 2009; Kilanczyk, Filipek et al. 2011). Prkca was down-regulated by GnRH-treatment. It was previously reported to function as a negative regulator of Wnt/ β-catenin signaling pathway, which phosphorylated N-terminal Ser/Thr residues of β-catenin and subsequently induced its degradation (Gwak, Cho et al. 2006; Gwak, Jung et al. 2009). All the above findings suggest that these significant altered proteins may play crucial roles in mediating crosstalk between Wnt signalling and GnRH signalling in the field of gonadotrope biology.

4.2.3 Validation of ICAT identification by Western blot

Among all the significantly altered protein, Cacybp was selected for downstream validation. The expression levels of Cacybp in biological triplicates were validated by western blot analysis. As shown in Figure 4.6, the expression level of Cacybp was down-

regulated by GnRH in all the biological replicates. The result is consistent with our ICAT identification.

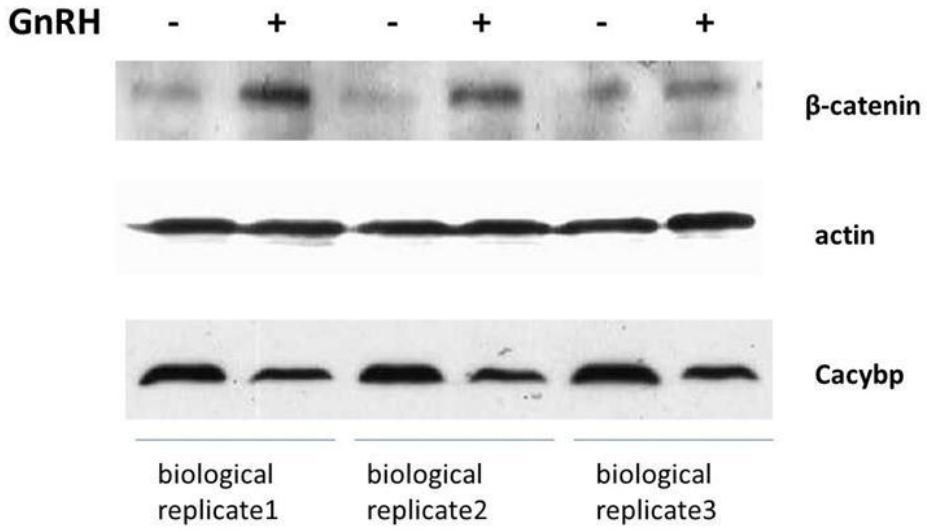


Figure 4.6 Validation of ICAT identification on selected proteins.

Western blot studies were conducted to show the Cacybp and β-catenin expression levels in 4 h mock-treated and GnRH-treated LβT2 cells. Actin was used as the loading control.

4.2.4 Investigation of intracellular expression of β-catenin upon GnRH-induction

Although β-catenin is the focus point of Wnt signaling, we, however, were not able to detect it in ICAT profiling. We therefore used other methods to investigate the intracellular expression of β-catenin. First, we used western blot analysis to compare the overall expression levels of β-catenin in 4 h mock-treated and GnRH-treated LβT2 cells, and observed an increase of the expression level of β-catenin upon treatment in two of the biological triplicates (Figure 4.6). Next we did immunofluorescence experiment to check the cellular distribution of β-catenin in 4 h mock-treated and GnRH-treated LβT2 cells (Figure 4.7), and showed that the protein level of β-catenin in the whole cell was significantly increased after GnRH-treatment, which is consistent with the western blot result. However, the increasing amount of β-catenin was mostly present in the cytoplasm. While there might be slight increase of β-catenin level in the nucleus, a more precise quantitative measurement will be needed to confirm it in the future.

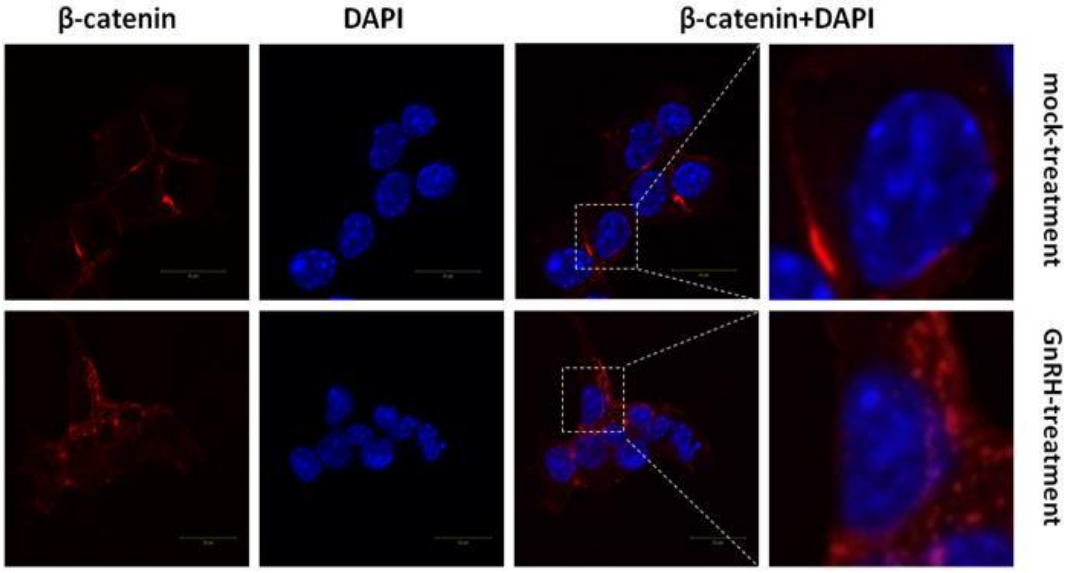


Figure 4.7 Immunofluorescence analysis for intracellular distribution of β-catenin after 4 h GnRH-induction in LβT2 cells.

LβT2 cells were seeded on pre-coated coverslips and serum starved for 12 h, and were exposed to 100 nM GnRH or mock for 4 h. Cells were fixed and then stained for β-catenin (red) and nuclei with DAPI (blue). Scale bar=10 μm.

4.2.5 Investigation of mechanisms mediating GnRH regulation on β-catenin

As we have found that β-catenin expression level is elevated by GnRH induction, we next investigate which mechanism mediates the GnRH regulation on β-catenin. So far it has been known that the activity of β-catenin can be regulated through two ubiquitination pathways, each of which is composed of different E3 ligase complexes (Logan and Nusse 2004; Fukushima, Zapata et al. 2006). The best characterized one was GSK-3β-dependent degradation of β-catenin (Kitagawa, Hatakeyama et al. 1999; Latres, Chiaur et al. 1999; Liu, Kato et al. 1999; Winston, Strack et al. 1999). GSK-3β is a well-known negative regulator of Wnt signaling. The phosphorylation of GSK-3β at Ser 9 induced by Wnt ligand activation has been suggested to result in the inhibition of GSK-3β activity, thus rescue β-catenin from degradation (Gardner, Maudsley et al. 2007). Hence we thought to check whether the phosphorylation status of GSK-3β is also regulated by GnRH. We treated LβT2 cells with mock or GnRH for 30 min, 2 h, and 4 h respectively, extracted the total proteins (described in Chapter 3) from each treatment, and then conducted western blot to detect both the GSK-3β and p-GSK-3β (Ser 9) proteins. As shown in Figure 4.8, we found that the expression level of Ser 9 phosphorylated GSK-3β showed not much difference in the 30 min GnRH-treated cells compared to the mock-treated control, while we did see the difference in the 4 h treatment group that upon 4 h GnRH-treatment the abundance of p-GSK-3β was increased compared to the 4 h mock-treated control. However interestingly, the relative abundance of p-GSK-3β in 4 h GnRH-treated cells was reduced compared to 30 min GnRH-treated cells, and the phenomenon was the same for the mock-treated controls, given that there are no abundance changes for total GSK-3β proteins. The finding indicates that GSK 3β-dependent pathway, through

modulating the phosphorylation at Ser 9, may not be the only pathway mediating GnRH effect on β-catenin in LβT2 cells.

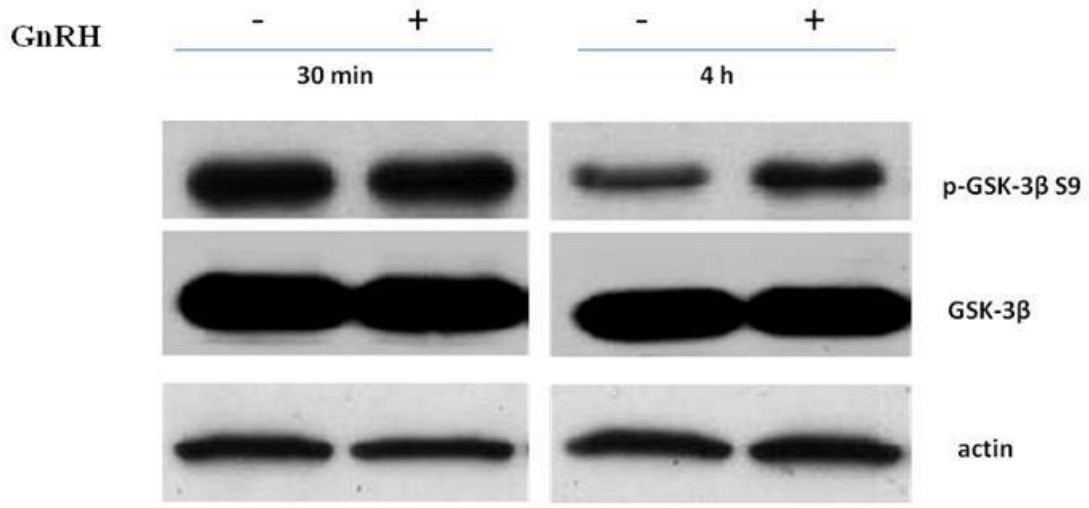


Figure 4.8 Western blot analysis of GnRH-induced Ser9 phosphorylation of GSK-3β in LβT2 cells.

LβT2 cells were treated with mock or GnRH for 30 min and 4 h respectively and the total proteins from each treatment were extracted and applied for western blot analysis. Both GSK-3β and p-GSK-3β (Ser 9) were detected. Actin was used as the loading control.

4.3 Discussion

Modulation of gonadotrope cell signaling by GnRH and its analogs has great application potential in the therapeutic field for treating the diseases such as infertility and gonadal hormone dependant tumors. However, so far, most knowledge of GnRH-induced regulation is at the transcriptional level, and the most well studied signaling pathway involved is MAPK signaling. To expand the current knowledge, we carried out this proteomics study. Since our initial aim is to identify regulatory proteins, which are mainly transcription factors and kinases and known to be low abundance proteins (Gygi, Rochon et al. 1999), we used the cICAT-based labeling technology. The top-ranked two molecular functions, which were identified by cICAT, were shown as phosphorylation and acetylation (Figure 4.3). Phosphorylation was known to be difficult to analyze by proteomic methods due to the small proportion of phosphorylated proteins among the whole protein complex. Our study demonstrated the effectiveness of the cICAT approach for detecting the low abundance proteins.

As mentioned just now, phosphorylation and acetylation are identified as two major molecular functions among all the GnRH-induced proteins. Acetylation is a crucial PTM process in regulation of histone, transcription factors, effecter proteins, molecular chaperons, and cytoskeleton proteins (Choudhary, Kumar et al. 2009). Although not many studies have been carried out in this field, acetylation has been implicated to be involved in the regulation of gonadotropins. It has been reported that upon GnRH induction, lysine residues of histone H3, H4 at the LHβ gene locus became acetylated (Melamed, Kadir et al. 2006), possibly through the active recruitment of histone acetyl transferases (HATs). The HAT, p300, was found to bind with LHβ gene promoter, and

the binding was enhanced by GnRH induction (Mouillet, Sonnenberg-Hirche et al. 2004). In addition, several transcription factors such as Sf-1, SRC-1, SRC-2, which activate LHβ and FSHβ in gonadotrope, were found to interact with HATs in other cell contexts (Jacob, Lund et al. 2001; Lund, Borud et al. 2002). GnRH-induced acetylation may become a new research area to explore in the future.

In this study, phosphorylation is identified as the top-ranked molecular function, which involves the most number of significantly altered proteins by GnRH induction. Phosphorylation and dephosphorylation of transcriptional factors and kinases have been widely reported in GnRH-induced MPAK pathways (Harris, Bonfil et al. 2002; Harris, Chuderland et al. 2003; Kraus, Benard et al. 2003; Lopez-Bergami and Ronai 2008), while in our study we found that several proteins involved in phosphorylation are also likely to regulate β-catenin, a crucial Wnt signaling effecter, as well as other mediators in Wnt signaling. The involvement of Wnt signaling in mediating the GnRH response was first published by Gardner's group, who found that β-catenin is a critical effecter mediating the GnRH induced production of LHβ gene (Salisbury, Binder et al. 2007), and possibly other gonadotropin genes (Salisbury, Binder et al. 2008). GnRH was also found to stimulate several known TCF target gens including *Jun*, *Fra1*, and *Myc* (Wurmbach, Yuen et al. 2001; Yuen, Wurmbach et al. 2002; Nateri, Spencer-Dene et al. 2005; Kikuchi, Kishida et al. 2006). However, how the GnRH signaling modulates the activity of β-catenin is not clear.

Here comes to the question: how the GnRH signal is transduced to β-catenin and TCF/LEF-dependent genes in gonadotrope cells? In the canonical Wnt signaling, regulation of β-catenin is always linked to a well-known GSk-3β dependent pathway, in

which a GSK-3β containing inhibition complex modulates the phosphorylation and degradation of β-catenin (Gordon and Nusse 2006; Kikuchi, Kishida et al. 2006). However in LβT2 cells, we observed increased expression level of β-catenin induced by GnRH but seems not through phosphor-inhibition of GSK-3β at Ser 9. Thus we conclude that in gonadotrope cells, GnRH regulation of β-catenin may not be through inhibiting GSK-3β, or through inhibiting GSK-3β but in a manner, independent of Ser 9 phosphorylation. Other than the GSk-3β-dependent pathway, the stability of β-catenin can be modulated by GSk-3β-independent mechanism. In this pathway, β-catenin degradation can be mediated by direct interaction with an ubiquitin ligase complex. Cacybp, which was found to be down-regulated by GnRH, is just one component of E3 ubiquitin ligase complex (Filipek 2006). Unlike the GSK-3β inhibition complex, Cacybp inhibition complex controls the degradation of β-catenin not through phosphorylation (Filipek 2006; Fukushima, Zapata et al. 2006). Furthermore, Cacybp/SIP was found as a novel phosphatase, which has the ability to dephosphorylate ERK1/2 (Kilanczyk, Filipek et al. 2011). As ERK phosphorylation is required for the activation of gonadotropin responsive genes, such as Nur77 (Bliss, Navratil et al. 2012), the down-regulation of Cacybp may also facilitate the expression of gonadotropin responsive genes. While the mechanisms of Cacybp regulation by GnRH is not clear, recent evidence indicated that it is likely regulated through GnRH-induced Ca^{2+} signalling pathway (Liang, Luo et al. 2007). Ddx17 and its homologous protein Ddx5 were reported to be transcriptional coactivators of several key transcriptional factors (Janknecht 2010; Fuller-Pace and Moore 2011). Ddx17 was found to be up-regulated by GnRH treatment. Ddx17 was previously found to act as a coactivator of β-catenin through forming a complex with β-

catenin in the nucleus and subsequently promote the β-catenin target gene expression (Shin, Rossow et al. 2007). As Ddx17 was up-regulated by GnRH treatment, it may therefore stimulate β-catenin-dependent transcription in LβT2 cells. The enhanced stability of Ddx17 and Ddx5 have been hypothesized to be induced by their posttranslational modification (Shin, Rossow et al. 2007). As overexpression of Ddx5 was found to occur simultaneously with the poly ubiquitination (Causevic, Hislop et al. 2001) and phosphorylation of Ddx5 (Yang, Lin et al. 2005). Thus, GnRH may also regulate the modifications of Ddx17 and consequently induces the expression of Ddx17. Prkca was previously reported to function as a negative regulator of Wnt/ β-catenin signaling pathway (Gwak, Cho et al. 2006; Gwak, Jung et al. 2009). Depletion of Prkca inhibited GSK-3β-independent phosphorylation of β-catenin, and consequently stabilized the β-catenin. Prkca was found to be down-regulated by GnRH in our study; therefore the β-catenin may consequently be stabilized by GnRH in gonadotrope. It may also explain our finding that p-GSK-3β Ser 9 is not required for regulating β-catenin level in LβT2 cells. Ppp2ca was down-regulated by GnRH treatment. Ppp2ca is the catalytic subunit of PP2A kinase (Janssens and Goris 2001), which was suggested to be essential for the degradation of β-catenin in *Xenopus*, and implicated to be the dominant inhibitor of Wnt signaling (Li, Yost et al. 2001). Later work done in a number of cell lines found that PP2A was required for the dephosphorylation of GSK-3β, which consequently enhanced the activity of GSK-3β and subsequently resulted in the degradation of β-catenin (Ivaska, Nissinen et al. 2002; Hildesheim, Belova et al. 2003; Lee, Seo et al. 2005). Furthermore Ser 9 of GSK-3β was suggested to be the target site by PP2A (Mitra, Menezes et al. 2012). Based on the proposed actions of the identified proteins, we plotted a model of the

new interactive pathways (Figure 4.9). These findings advanced the current knowledge of GnRH signaling in gonadotrope cell line from regulating MAPK cascades to cross talk with Wnt signaling. However, further works will be needed to prove this proposed model.

Last but not least, we have to acknowledge that this ICAT-based proteomic profiling, though provided useful information in signaling pathway, only recognize cysteine-containing peptides, thus it inevitably missed out quite a number of non-cysteine contained proteins. Therefore a more comprehensive labeling method, such as iTRAQ is needed to complementary this study. Furthermore, translocation is often concurrent with the regulatory proteins, identification of the localization changes of proteins in the GnRH signaling may help to reveal crucial events involved in the regulation of gonadotropin production. This part of the study will be presented in the next chapter.

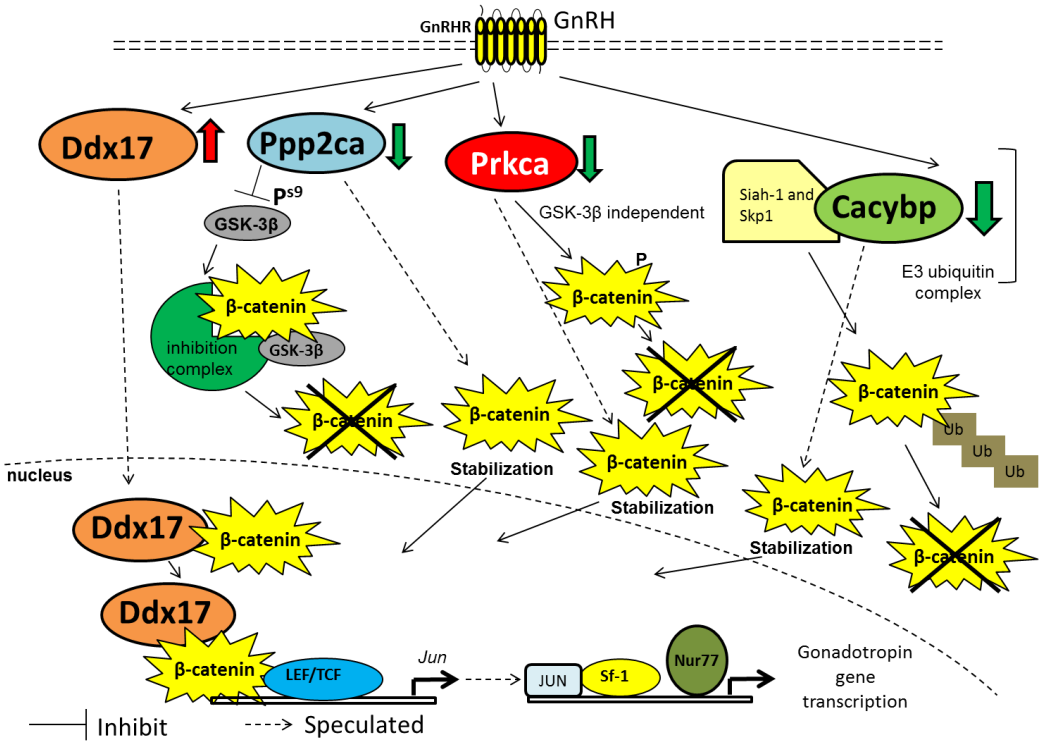


Figure 4.9 Proposed model for cross talk between GnRH signaling and Wnt signaling.

β-catenin, a crucial mediator of Wnt signaling, is perhaps stabilized by GnRH signaling through two mechanisms, which are shown as stabilization (a) and stabilization (b). Stabilization (a) is a β-catenin phosphorylation-dependent mechanism, where the phosphorylation of β-catenin, which results in the degradation, is modulated by either GSK-3β Ser 9 dependent-mechanism or GSK-3β Ser 9-independent mechanism. Prkca, which facilitates the phosphorylation and degradation of β-catenin was down-regulated by GnRH, therefore, possibly stabilizing β-catenin. Ppp2A, a phosphatase of GSK-3β Ser 9, was down-regulated by GnRH, which may therefore stabilizing β-catenin through inactivating GSK-3β. Stabilization (b) is a β-catenin phosphorylation-independent mechanism, which is mediated by Cacybp-containing E3 ubiquitin complex. Cacybp is a negative regulator of β-catenin stabilization. The down-regulation of Cacybp by GnRH, possibly through Ca2+ signaling, may thus result in the stabilization of β-catenin. Besides the possibility of stabilizing β-catenin in gonadotrope, down-regulation of Cacybp may also enhance the activity of ERK 1/2 kinase, whereby facilitating the PKC pathway. Except for modulating β-catenin stability, GnRH may also modulate β-catenin transactivation activity through up-regulating Ddx 17, the co-activator of β-catenin.

Chapter 5. iTRAQ proteomic profiling of the cellular response induced by GnRH in gonadotrope cell line

5.1 Introduction

GnRH serves as a key hormone to regulate reproduction of vertebrates. It is released from hypothalamus in a pulsatile manner and functions through GnRHR binding to induce the activation of multiple downstream signalling pathways, which eventually stimulate the synthesis and secretion of pituitary gonadotropins LH and FSH (Naor 2009). The variations in GnRH pulse frequency are associated with different physiological conditions as well as different developmental stages during the productive life. GnRH secretion is suppressed during the pre-pubertal period while an augmentation of GnRH pulse occurs at the onset of pubertal maturation. In adult males, the pulse of GnRH secretion is moderately consistent whereas in adult women it changes during the ovulatory menstrual cycle (Marshall, Dalkin et al. 1993). GnRH pulse frequency gradually increases during the follicular phase, which induces a surge in gonadotropin secretion and consequently causes ovulation (Ferris and Shupnik 2006). The variation of GnRH pulses is also associated with pathologic conditions. Polycystic ovarian syndrome (PCOS) is the most common cause of infertility in women affecting 10% of the population (Ehrmann 2005; Azziz, Carmina et al. 2009). In patients with PCOS, reproductive cycle is disrupted by consistently elevated LH level and depressed FSH level, leading to an increase in androgen production by ovarian theca cells (Ehrmann 2005). The synthesis and secretion of gonadotropins, essential for maintaining normal physiological conditions, are dependent on GnRH pulse frequency, perturbation of which induces infertility (Gharib, Wierman et al. 1990). *In vivo* studies have shown that the pulsatile stimulation by GnRH is more effective than sustained stimulation. The latter

causes chemical contraception and has been developed into the treatment of hormone-dependent cancers (Conn and Crowley 1994).

Variation in GnRH pulses leads to divergent LH and FSH secretion, rendering a mechanism by which a single hormone induction can cause differential changes in two distinct hormones released from the same gonadotrope cell. The alteration of GnRH pulsatility during the luteal and early follicular phases of the ovulatory cycle, favors the production of FSHβ, providing the increased pituitary FSH secretion essential for the recruitment and selection of the maturing ovum (REAME, SAUDER et al. 1984; Haisenleder, Dalkin et al. 1991; Kaiser, Jakubowiak et al. 1997; Burger, Haisenleder et al. 2004; Ferris and Shupnik 2006). As noted above, GnRH pulsatility to the pituitary gonadotrope is crucial for the physiology and therapeutic manipulation of the reproductive system. However, the underlying mechanism driving the differential regulation of gonadotropins, although likely rests on the ability of the gonadotrope to decipher different GnRH input patterns (Burger, Haisenleder et al. 2004; Ferris and Shupnik 2006; Ruf, Park et al. 2006), is still unclear.

In last chapter, we utilized cICAT-based proteomics approach to compare differentially expressed proteins in whole cell lysate from mock-treated and GnRH-treated LβT2 cells. We identified a few proteins involved in PTMs and other signalling pathways other than MAPK cascades, which revealed the novel regulatory roles of GnRH and novel pathways GnRH may target. However, cICAT reagent can only label the proteins which carry cysteine-residue, thus it will inevitably miss out the non-cysteine containing proteins. Furthermore, cICAT-labeling only allows two-sample comparison, which limits the multiplex design of the experiments (Zieske 2006). These limitations may affect the

interpretation of GnRH signalling from a comprehensive perspective. To overcome these disadvantages, we used an alternative proteomic method - iTRAQ to perform comparative profiling of the nuclear proteomes of the mock-treated and GnRH-treated LβT2 cells. Rather than just a repetitive study, our iTRAQ study changed the GnRH treatment from continuous treatment to pulse treatment, which better represents the physiological condition (Lawson, Tsutsumi et al. 2007).

Our study revealed that proteins involved in RNA processing, translation and chromosome organization were differentially regulated by GnRH pulse frequency. Our findings provide preliminary evidence showing the novel regulatory role of GnRH on gonadotropin. Furthermore, our findings suggest the involvement of Ca^{2+} signaling in decoding the GnRH pulse frequency.

5.2 Results

5.2.1 Analysis of the influence of differential GnRH frequency on nuclear proteome of the LβT2 cell line

To better understand how GnRH signalling is decoded to differentially regulate LHβ and FSHβ gene expression, we carried out iTRAQ proteomics profiling of nuclear proteins alteration in GnRH-treated LβT2 cells. GnRH treatment was done for 30 min and 4 h, respectively. 30 min treatment represented the short frequency, whereas 4 h treatment mimicked the long frequency. After treatment, nuclear proteins were isolated from individual samples and subjected to 8-plex iTRAQ labeling. The details of the sample preparation were described in the chapter 3. The design of the iTRAQ labeling experiment is illustrated in Figure 5.1.

A strict cutoff threshold with unused protein score ≥ 1.3, corresponding to 95% C.I., was used as the protein identification criteria. Under this criteria, the 8-plex iTRAQ experiment identified 2733 proteins (Supplementary soft copy). Among these, 86 % proteins were identified with ≥ 2 - peptide (95% C.I.) matches, indicating high quality of the iTRAQ data. Next, the cutoff threshold of iTRAQ ratio was determined by a biological replicate method, which was proposed by (Gan, Chong et al. 2007). As shown in Figure 5.2, the final cut-off for both 30 min and 4 h GnRH-treatment were fixed at 1.5 fold (±50 % variation), corresponding to ≥ 1.5 for up-regulation and ≤ 0.67 for down-regulation.

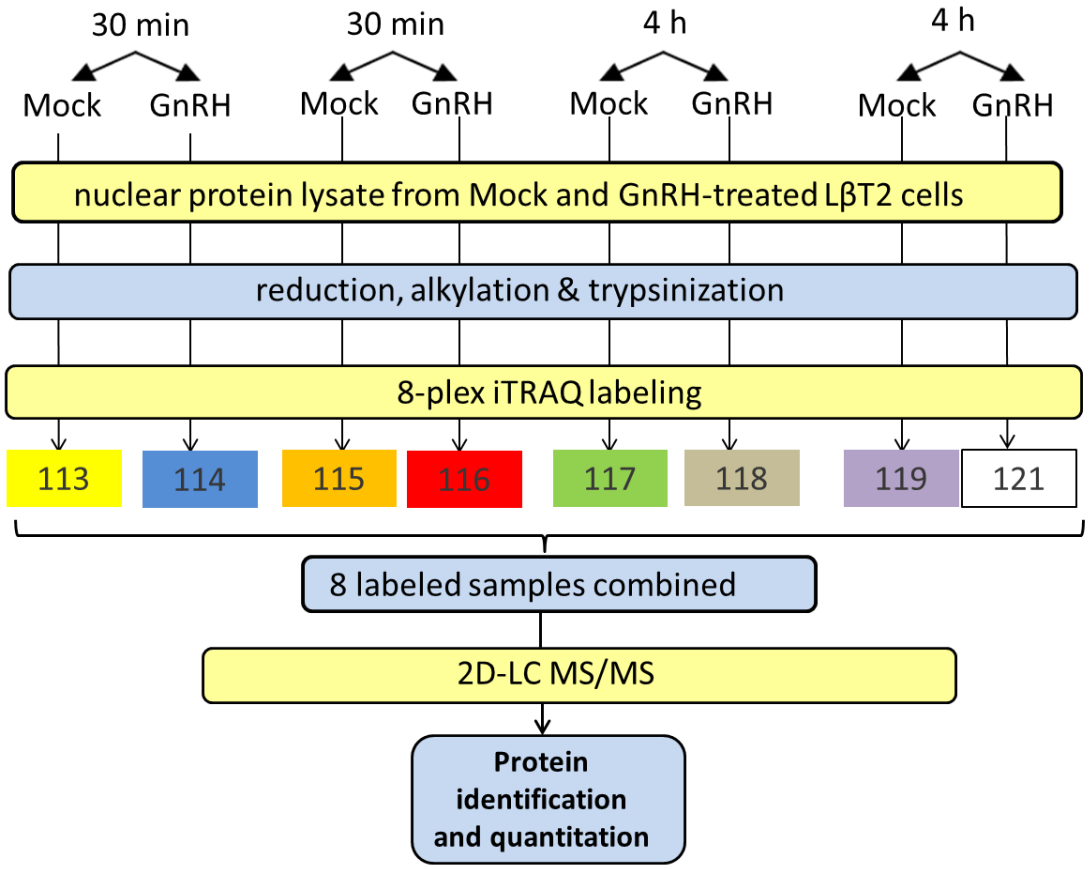


Figure 5.1 Schematic representation of the workflow of the iTRAQ–based proteomic profiling.

8-plex iTRAQ labeling of the nuclear cell lysates from 30 min and 4 h mock-treated and GnRH-treated LβT2 cells was carried out in duplicates. Duplicates from 30 min mock-treated nuclear cell lysates were labeled with reagents113 and 115, and those from 30 min GnRH-treated were labeled with reagents114 and 116. Similarly, duplicates from 4 h mock-treated nuclear cell lysates were labeled with reagents117 and 119, and those from 4 h GnRH-treated were labeled with reagents118 and 121.

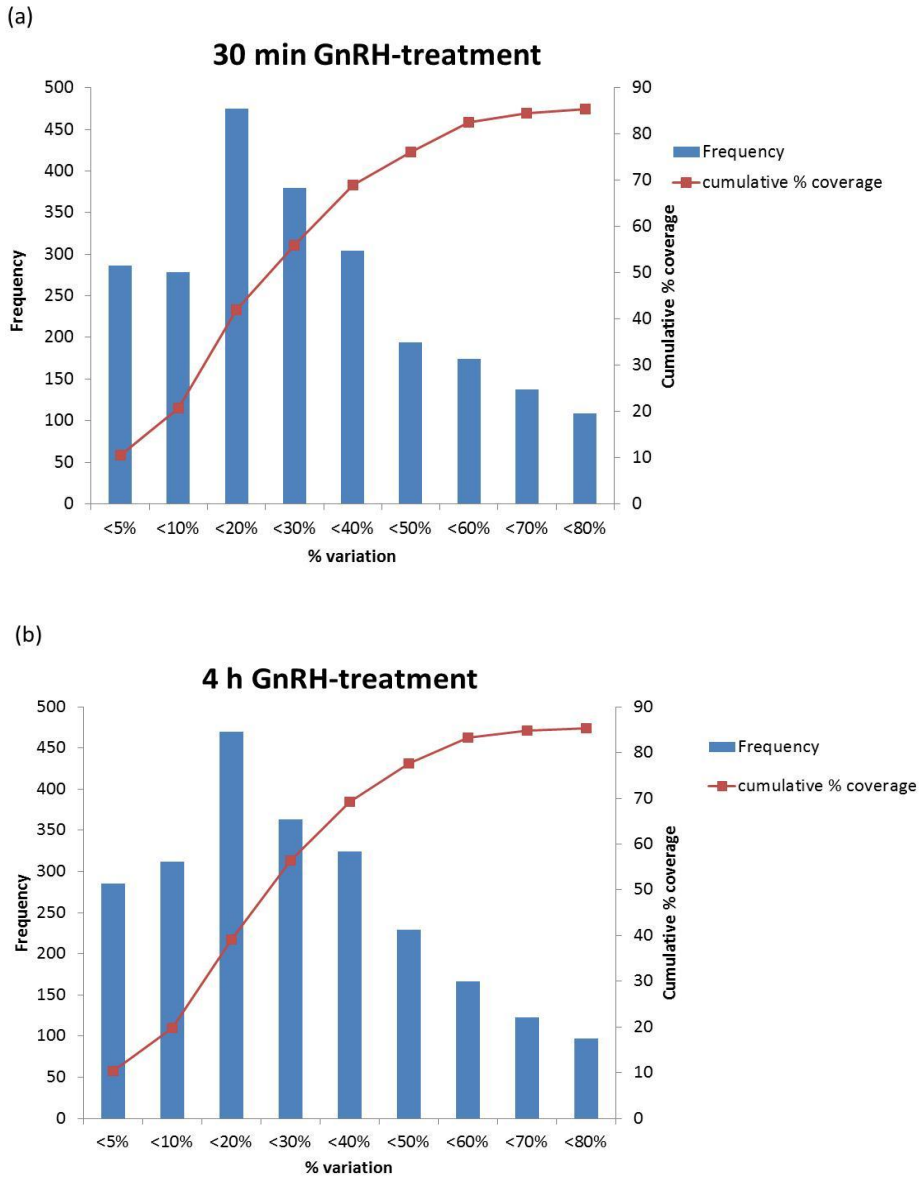


Figure 5.2 Determination of the cut-off ratio for iTRAQ experiments.

(a) 30 min GnRH-treatment (b) 4 h GnRH-treatment. The cut-off ratio were determined by calculating the percentage variation of iTRAQ ratios of each protein from biological replicates; the resulting values were shown in horizontal axis. Next the numbers of the proteins whose iTRAQ ratio fell into the indicated percentage variation were counted; the numbers were shown in vertical axis. Then graphs were plotted and about 48% variation corresponding to 88% coverage were observed in both the 30min and 4 h GnRH-treatment.

This cut-off threshold was applied to both replicates of 30 min GnRH-treatment as well as 4 h GnRH-treatment, and only the proteins met the criteria in both replicates were selected. As a result, there were 108 proteins in 30 min GnRH-treatment, and 311 proteins in 4 h GnRH-treatment, whose expression levels were considered to be significantly altered. The corresponding log10 ratios of these significantly altered proteins were plotted to demonstrate the correlation between the two replicates for each treatment. As shown in Figure 5.3, the correlation coefficients were 0.82 and 0.77 for 30 min and 4 h treatments respectively, indicating acceptable variations in both datasets. Interestingly, among these significantly altered proteins, only 18 proteins were regulated by both 30 min and 4 h treatments with the same trend. Furthermore, 5 proteins showed significantly opposite trends of alteration in the two different GnRH frequency treatments (Figure 5.4). These findings implicated that different GnRH frequency does possibly cause varied cellular events in the gonadotrope cells.

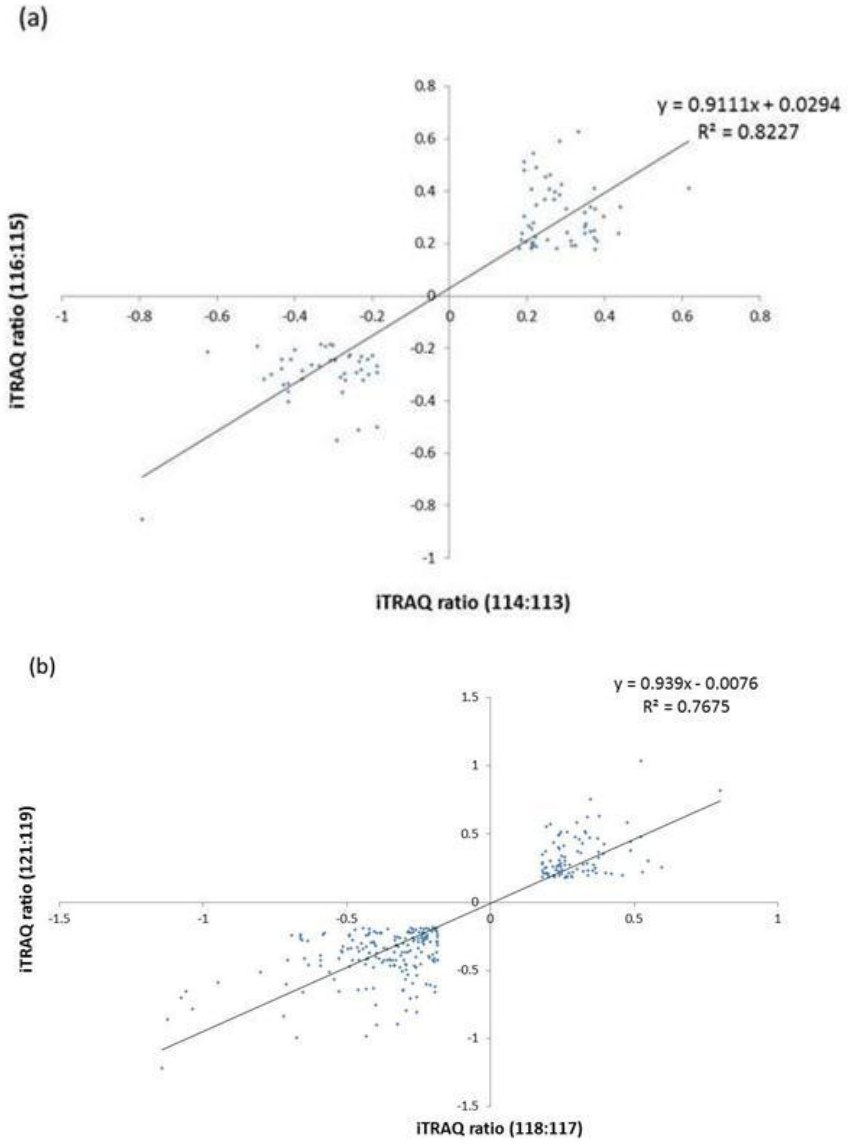


Figure 5.3 Correlation of iTRAQ ratios of the significantly altered proteins by GnRH-treatment between duplicates.

The ratios used to plot the figures were the $\log_{10}$ ratios. (a) Correlation between duplicates of 30 min GnRH-treatments. (b) Correlation between duplicates of 4 h GnRH-treatments.

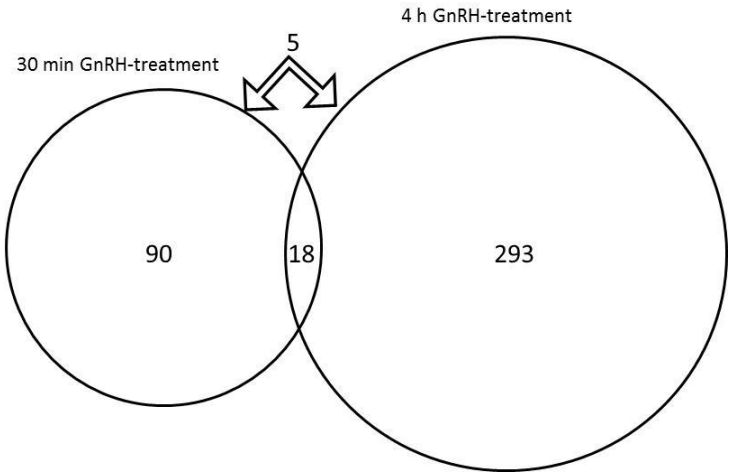


Figure 5.4 Distribution of the significantly regulated proteins by 30 min GnRH-treatment and 4 h GnRH-treatment.

108 proteins in 30 min treatment and 311 proteins in 4 h treatment were identified as the significantly regulated proteins by GnRH. Among these, only 18 proteins were regulated by both 30 min and 4 h treatments with the same trend. Furthermore, 5 proteins showed significantly opposite trends of alteration in the two different GnRH frequency treatments.

5.2.2 Gene ontology study of the significantly altered proteins by GnRH induction

5.2.2.1 Functional characterization of the overall proteins significantly regulated by GnRH induction

To get a better insight into the functional relationship of the significantly altered proteins to the regulation of gonadotropins, we performed the gene ontology study. First, we applied IPA core analysis to all the significantly altered proteins. The top 3 molecular and cellular functions were RNA post-transcriptional modification, protein synthesis, and cellular assembly and organization (Figure 5.5). These findings were consistent with the biological topic of this study--regulation of gonadotropins.

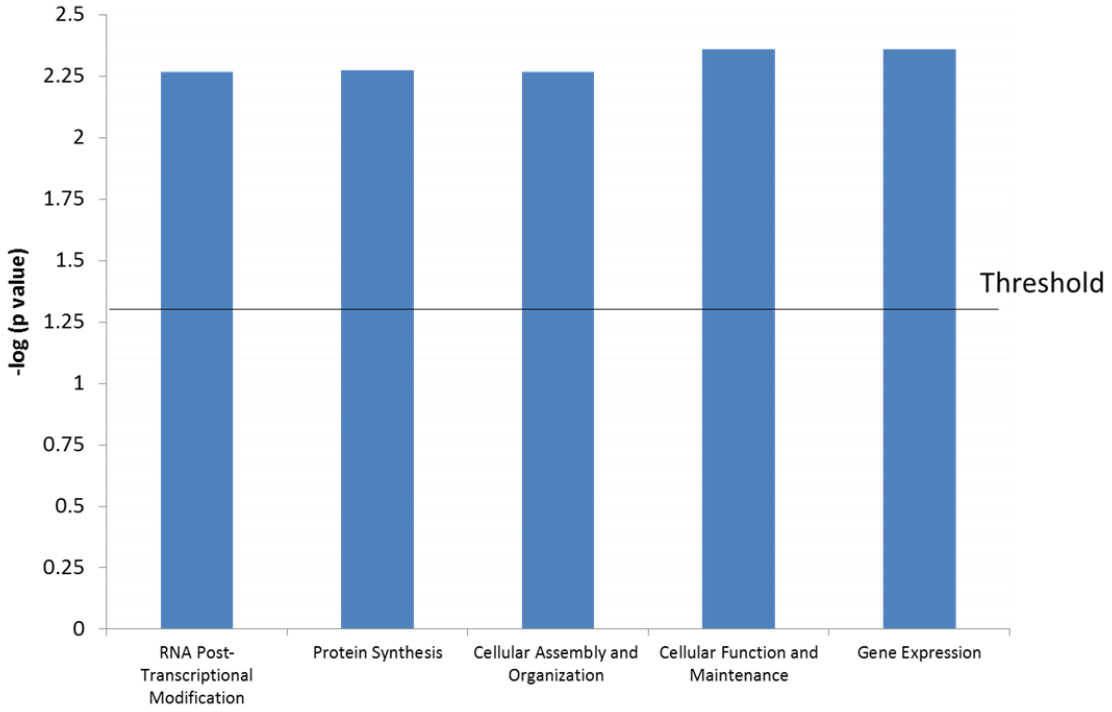


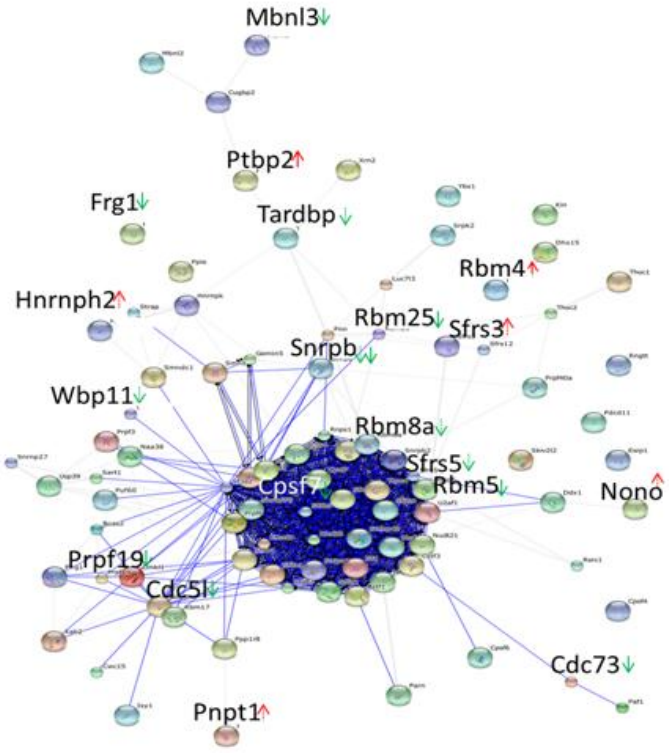
Figure 5.5 Top ranked molecular and cellular functions of all the significantly altered proteins.

Top ranked molecular and cellular functions of all the 396 proteins, which combined both of the 30 min- and 4 h-GnRH regulated proteins, were derived from the IPA core analysis. (Graph was redrawn based on the IPA output file)

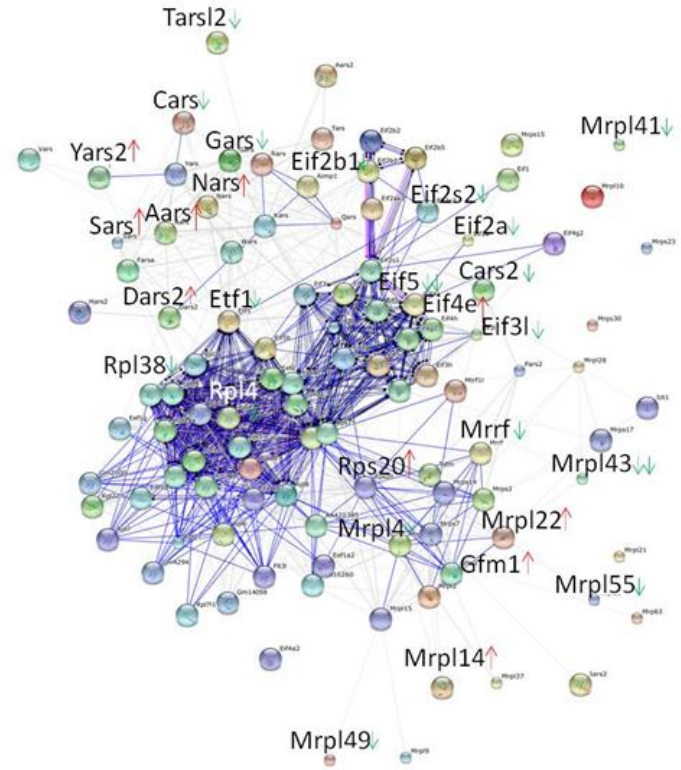
To investigate the mechanisms involved in the above molecular and cellular functions, we utilized STRING software to analyze the protein-protein interaction networks, which may help to discover the underlying mechanism. We entered the protein IDs of the significantly altered proteins into STRING open source software and looked for the top ranked biological processes. Among these, we selected three most relevant biological processes, which were mRNA processing, translation and chromosome organization, for further analysis. Next we inputted all the 2733 identified protein IDs to STRING, and only look for the proteins which were involved in the three most relevant biological

processes. After sorting these IDs, we analyzed the protein-protein interaction of the overall proteins involved in each of the three biological processes. These protein networks were illustrated in Figure 5.6.

(a)


Mbnl3
Ptbp2
Frg1
Tardbp
Rbm4
Hnrnph2
Rbm25
Sfrs3
Snrpb
Wbp11
Rbm8a
Sfrs5
Cpsf7
Rbm5
Nono
Prpf19
Cdc5l
Cdc73
Pnpt1

(b)


Tarsl2
Cars
Mrpl41
Gars
Yars2
Eif2b1
Nars
Eif2s2
Aars
Sars
Eif2a
Cars2
Eif5
Dars2
Etf1
Eif4e
Eif3l
Rpl38
Rpl4
Mrrf
Rps20
Mrpl43
Mrpl22
Mrpl4
Gfm1
Mrpl55
Mrpl14
Mrpl49

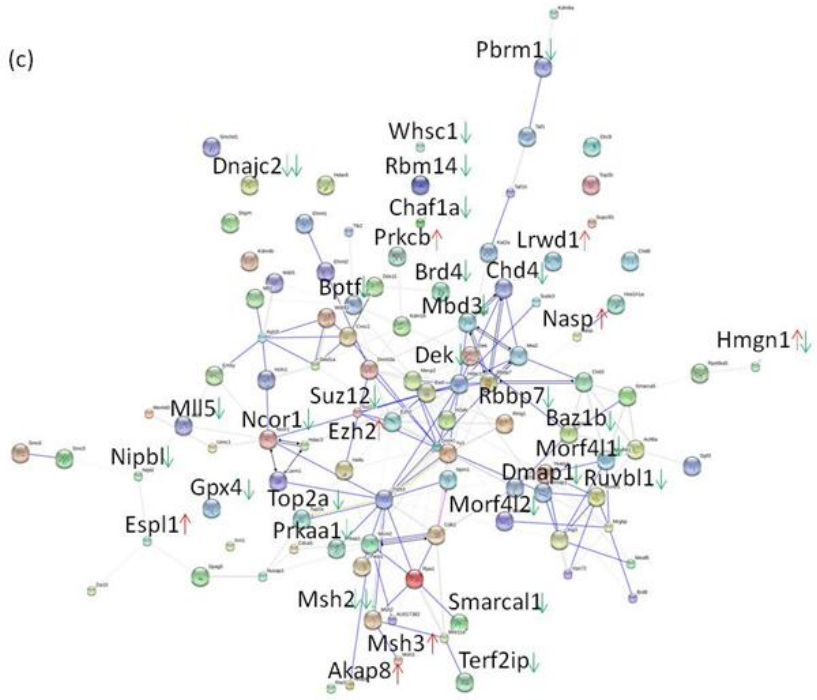


Figure 5.6 Analysis of interaction networks of (a) proteins involved in mRNA processing; (b) proteins involved in translation; (c) proteins involved in chromosome organization using STRING.

The overall 2733 identified protein IDs were analyzed by STRING to screen for the interaction networks involved in (a) mRNA processing, (b) translation, and (c) chromosome organization. The analyses were carried out under the interactive network mode and the action view. Densely connected networks were clustered and color coded. (red ↑ stands for up-regulation, green ↓ stands for down-regulation; dashed arrows refer to 30 min-GnRH treatment, solid arrows refer to 4 h-GnRH treatment)

Proteins involved in mRNA processing

A recent paper reported that transcription and splicing are physically and functionally coupled (Montes, Becerra et al. 2012). Although GnRH-induced transcriptional regulation of gonadotropins has been studied extensively, little work has been done to investigate the role of GnRH in regulating splicing. In our study, we identified 19 proteins which were known to participate in mRNA processing (Figure 5.6 (a)), were regulated by GnRH-treatment. Table 5.1 shows the list of these proteins, which were further grouped according to the sub-functions including serine –arginine proteins, RNA binding motif (Rbm) proteins, spliceosome component proteins and pre-mRNA processing proteins. Six out of the 19 proteins (31.6 %) were up-regulated by GnRH treatment, while the rest (68.4 %) were down-regulated. Among these proteins, only one protein, small nuclear ribonucleoprotein associated protein B (Snrpb), was down-regulated by both 30 min and 4 h GnRH treatments.

Table 5.1 Proteins involved in mRNA processing.

(↑↑ stand for >1.5, ↑ stand for > 1.3; ↓↓ stand for <0.67, ↓ stand for <0.77)

unused score	% seq cov	accession number	name	iTRAQ ratio (30 min GnRH)		Exp Pat.	iTRAQ ratio (4 h GnRH)		Exp Pat.
				114:113	116:115		118:117	121:119	
Serine-arginine proteins									
2.34	49.1	IPI00830743.1	Srsf5 serine/arginine-rich splicing factor 5	0.4786	0.6427	↓↓	1.0666	0.8017	
15.55	58.5	IPI00129323.1	Srsf3 Isoform Long of Serine/arginine-rich splicing factor 3	2.355	2.5823	↑↑	2.8054	0.5808	
RNA binding motif (Rbm)proteins									
10	34.4	IPI00309195.2	Rbm14;Rbm4 Isoform 1 of RNA-binding protein 4	1.7865	1.0666		1.7378	1.5704	↑↑
28.74	33.9	IPI00928541.1	Rbm25 Isoform 2 of RNA-binding protein 25	0.6243	0.9039		0.4169	0.5916	↓↓
6.97	21.9	IPI00798508.1	Rbm5 Isoform 2 of RNA-binding protein 5	1.0765	0.9727		0.7178	0.7516	↓
5.13	32.8	IPI00759967.1	B020018G12Rik hypothetical protein LOC545388	0.4169	0.6792	↓	0.6252	1.7865	
spliceosome component proteins									
7.78	59.9	IPI00108143.1	Hnrnph2 Heterogeneous nuclear ribonucleoprotein H2	2.3768	1.5136	↑↑	2.8054	0.4831	
26.99	48	IPI00480507.1	Prpf19 Isoform 1 of Pre-mRNA-processing factor 19	0.5861	1.4454		0.4487	0.3733	↓↓
8.67	42.4	IPI00114052.1	Snrpb Small nuclear ribonucleoprotein-associated protein B	0.4656	0.6546	↓↓	0.3281	0.4613	↓↓
pre-mRNA processing proteins									
70.48	67.7	IPI00320016.7	Nono Isoform 1 of Non-POU domain-containing octamer-	1.3062	2.1478	↑	2.3335	0.3373	

			binding protein						
7.94	24	IPI00263944.4	Mbnl3 Muscleblind-like protein 3	0.9727	0.9908		0.6918	0.6546	↓
9.84	31.4	IPI00123333.5	Wbp11 WW domain-binding protein 11	0.5916	0.7178	↓	0.5546	0.8395	
2	41.8	IPI00121758.1	Tardbp TAR DNA-binding protein 43	0.7447	0.2938	↓	1.0375	0.5248	
10.14	26.2	IPI00170345.1	Cdc73 Parafibromin	0.5916	2.729		0.5152	0.4325	↓↓
39.66	53.9	IPI00284444.5	Cdc5l Cell division cycle 5-related protein	0.8318	1.2246		0.5702	0.7447	↓
6	31.6	IPI00751759.1	Ptbp2 Isoform 1 of Polypyrimidine tract-binding protein 2	1.2706	0.9908		1.3428	1.3677	↑
13.31	31.4	IPI00720208.1	Cpsf7 Isoform 2 of Cleavage and polyadenylation specificity factor subunit 7	0.9376	0.7656		0.4656	0.7311	↓
6	31.8	IPI00311968.1	Frg1 Protein FRG1	1.0471	0.7311		0.7447	0.7178	↓
12.56	31	IPI00321923.5	Pnpt1 Isoform 1 of Polyribonucleotide nucleotidyltransferase 1, mitochondrial	1.6749	1.4588	↑	0.9462	0.9727	

Proteins involved in translation

Except for transcriptional regulation of gonadotropins, GnRH was once reported to enhance the translation of LHβ gene (Nguyen, Santos et al. 2004). However, no other group published any findings about translational regulation of gonadotropins induced by GnRH. In this study, we found 28 proteins which were known to be involved in translation were regulated by GnRH (Figure 5.6 (b)). Table 5.2 shows the list of these proteins, which were further divided into sub-functional groups including eukaryotic translation initiation factors, aminoacyl-tRNA synthetases, ribosomal proteins, and other proteins involved in translation. Eighteen out of 28 proteins (64.3 %) were down-regulated by GnRH treatment, while the rest (35.7 %) were up-regulated. Among these proteins, Eukaryotic translation initiation factor 5 (Eif5) and 39S ribosomal protein L43 (Mrpl43) were down-regulated by both 30 min and 4 h GnRH-treatments.

Table 5.2 Proteins involved in translation.

(↑↑ stand for >1.5, ↑ stand for > 1.3; ↓↓ stand for <0.67, ↓ stand for <0.77)

unused score	% seq cov	accession number	name	iTRAQ ratio (30 min GnRH)		Exp Pat.	iTRAQ ratio (4 h GnRH)		Exp Pat.
				114:113	116:115		118:117	121:119	
eukaryotic translation initiation factors									
6.46	27.7	IPI00187443.1	Eif5;LOC100047658 Eukaryotic translation initiation factor 5	0.4169	0.52	↓↓	0.3373	0.6252	↓↓
10.54	38.9	IPI00119806.6	Eif2a Isoform 1 of Eukaryotic translation initiation factor 2A	0.2559	0.7516	↓	0.6546	0.8551	
5.6	15.3	IPI00463573.3	Eif3l Eukaryotic translation initiation factor 3 subunit L	0.5248	0.492	↓↓	1.2359	2.2491	
35.57	70.7	IPI00116302.3	Eif2s2 Eukaryotic translation initiation factor 2 subunit 2	0.9817	1.0666		0.3597	0.4571	↓↓
9.35	26.2	IPI00116761.1	Eif2b1 Translation initiation factor eIF-2B subunit alpha	0.9727	1.6596		0.5754	0.5598	↓↓
3.01	33.6	IPI00119057.1	Eif4e Eukaryotic translation initiation factor 4E	1.1482	0.7798		2.7542	1.5136	↑↑
aminoacyl-tRNA synthetases									
2.02	18.1	IPI00229726.2	Tarsl2 Probable threonyl-tRNA synthetase 2, cytoplasmic	0.3467	0.5012	↓↓	1.2589	0.7447	
4	16.3	IPI00112555.3	Gars Glycyl-tRNA synthetase	0.4406	0.5445	↓↓	1.3183	0.2148	
12.15	24.4	IPI00273767.3	Dars2 Aspartyl-tRNA synthetase, mitochondrial	1.4997	2.8054	↑	1.0093	1.1912	

4.46	19.1	IPI00410740.1	Cars Isoform 2 of Cysteinyl-tRNA synthetase, cytoplasmic	0.7244	0.7244	↓	1.0093	1.1912	
2	14	IPI00459324.1	Cars2 Putative uncharacterized protein	0.787	1.0471		0.5445	0.6855	↓
6.66	23.2	IPI00469317.4	Sars Seryl-tRNA synthetase, cytoplasmic	0.5012	1.3932		1.9055	1.4723	↑
4.01	15.3	IPI00396833.1	Yars2 Tyrosyl-tRNA synthetase, mitochondrial	1.2706	1.0186		1.7378	2.0324	↑↑
9.59	28.5	IPI00321308.4	Aars Alanyl-tRNA synthetase, cytoplasmic	0.879	0.929		2.2284	5.6494	↑↑
7.94	33	IPI00223415.4	Nars Asparaginyl-tRNA synthetase, cytoplasmic	0.9462	0.9376		2.1086	1.6749	↑↑
ribosomal proteins									
10.98	58	IPI00323819.3	Rps20 40S ribosomal protein S20	0.5808	3.0479		2.5119	1.6444	↑↑
2	41.4	IPI00331121.4	Rpl38 60S ribosomal protein L38	0.7447	1.3062		0.4406	0.6368	↓↓
37.57	55.6	IPI00111412.3	Rpl4 60S ribosomal protein L4	0.3767	1.3677		0.5495	0.6252	↓↓
2	9.6	IPI00131988.1	Mrpl49 39S ribosomal protein L49, mitochondrial	0.6982	0.7516	↓	0.3945	1.0375	
4.3	17.9	IPI00133778.1	Mrpl14 39S ribosomal protein L14, mitochondrial	1.3428	1.4723	↑	1.2823	0.6486	
4.92	32	IPI00225318.3	Mrpl22 39S ribosomal protein L22, mitochondrial	1.6596	1.3428	↑	0.9908	1.0186	
4.03	35.4	IPI00121359.4	Mrpl4 39S ribosomal protein L4, mitochondrial	0.6194	0.5012	↓↓	0.3597	1.1482	

5.61	42.1	IPI00761722.2	Mrpl43 39S ribosomal protein L43, mitochondrial	0.5754	0.5105	↓↓	0.5916	0.5346	↓↓
5.77	48.9	IPI00378520.4	Mrpl41 39S ribosomal protein L41, mitochondrial	0.863	0.3133		0.6486	0.6486	↓↓
2	26	IPI00648969.2	Mrpl55 Isoform 1 of 39S ribosomal protein L55, mitochondrial	1.1912	1.1066		0.4742	0.7379	↓
other proteins involved in translation									
10.03	35.1	IPI00109501.1	Mrrf Ribosome-recycling factor, mitochondrial	0.9817	1.0765		0.5346	0.6368	↓↓
9.76	23.1	IPI00312468.5	Etf1 Eukaryotic peptide chain release factor subunit 1	0.5012	0.6486	↓↓	0.8318	1.0471	
19.95	31.3	IPI00230283.5	Gfm1 Elongation factor G, mitochondrial	1.3804	1.5276	↑	1.1482	0.6194	

Proteins involved in chromosome organization

A total number of 40 GnRH-regulated proteins were involved in chromosome organization (Figure 5.6 (c)). 35 proteins (87.5 %) were regulated by 4 h GnRH-treatment while only 8 proteins (20 %) were regulated by 30 min GnRH-treatment. Msh2 and Dnajc2 Uncharacterized proteins were found up-regulated by both 30 min and 4 h GnRH-treatments. However, Hmgn1 was found up-regulated by 30 min while down-regulated by 4 h GnRH-treatment. They were sub-divided to those involved in PTMs of N-terminal tails, those having structural roles in chromatin, those involved in DNA binding, and those involved in other functions (Table 5.3). Interestingly, over 50 % of the proteins which were involved in the chromosome organization were related to PTMs of the N-terminal tails, and the majority of these proteins were regulated by 4 h GnRH-treatment. PTMs of the N-terminal tail of each core histone, which include acetylation, methylation, phosphorylation, ubiquitylation and sumoylation, have been found to play crucial roles in the regulation of cellular processes through modulating the chromatin structures (Kooistra and Helin 2012). Among all the modifications, histone deacetylation has been recently studied in the gonadotrope cells (Lim, Luo et al. 2007; Melamed 2008). It was found that the repression of FSHβ gene expression caused by HDACs can be overcomed by GnRH. Besides histone deacetylation, acetylation has also been implicated to be involved in the regulation of gonadotropins (Melamed, Kadir et al. 2006), which has been discussed in Chapter 4.

Table 5.3 Proteins involved in chromosome organization.

(↑↑ stand for >1.5, ↑ stand for > 1.3; ↓↓ stand for <0.67, ↓ stand for <0.77)

unused score	% seq cov	accession number	name	iTRAQ ratio (30 min GnRH)		Exp Pat.	iTRAQ ratio (4 h GnRH)		Exp Pat.
				114:113	116:115		118:117	121:119	
proteins involved in PTMs of N-terminal tails									
5.04	15.1	IPI00831095.1	Ezh2 Uncharacterized protein	2.4889	2.0137	↑↑	1.3932	0.912	
8.02	17.9	IPI00762411.2	Whsc1 Isoform 1 of Probable histone-lysine N-methyltransferase NSD2	1.0864	0.8954		0.5495	0.3311	↓↓
2.03	16.9	IPI00660988.3	Mll5 Isoform 1 of Histone-lysine N-methyltransferase MLL5	1.1169	0.8551		0.6546	0.5598	↓↓
4.72	67.7	IPI00338745.4	LOC100044391;Hmgn1 Non-histone chromosomal protein HMG-14	1.556	3.02	↑↑	0.0871	0.2228	↓↓
10.08	29.5	IPI00317722.3	Dmap1 Isoform 1 of DNA methyltransferase 1-associated protein 1	1.2823	0.9817		0.631	0.413	↓↓
69.27	36.8	IPI00857777.1	Chd4 Uncharacterized protein	1.2359	1.3305		0.1941	0.2512	↓↓
20.31	32.4	IPI00652110.1	Brd4 Uncharacterized protein	1.1482	0.9817		0.7447	0.6668	↓
14.3	44.4	IPI00830720.1	Morf4l2 Uncharacterized protein	0.7047	1.3062		0.5445	0.6668	↓
12	24.3	IPI00278600.1	Morf4l1 Isoform 1 of Mortality factor 4-like protein 1	1.1169	1.1695		0.52	0.6026137	↓↓

4	18.6	IPI00720110.2	1600027N09Rik Uncharacterized protein	0.6982	0.5445	↓	0.492	0.7943	
13.95	22.7	IPI00274795.4	Ncor1 Uncharacterized protein	1.2823	0.8166		0.4742	0.4786	↓↓
100.52	52	IPI00469323.3	Dnmt1 DNA (cytosine-5)-methyltransferase 1 isoform 2	0.929	0.7178		0.2655	0.597	↑↑
10.39	35.8	IPI00122698.1	Rbbp7 Histone-binding protein RBBP7	1.2589	0.7244		0.7244	0.7447	↓
27.54	33.4	IPI00857769.1	Pbrm1 Uncharacterized protein	0.9376	0.9817		0.2965	0.3837	↓↓
16.44	28.1	IPI00404707.1	Rbm14 Isoform 1 of RNA-binding protein 14	1.0471	1.0471		0.5598	0.4613	↓↓
24.43	54.8	IPI00127415.1	Npm1 Nucleophosmin	0.631	1.1912		0.2333	0.3467	↓↓
10	28.7	IPI00396676.1	Suz12 Polycomb protein Suz12	0.9204	0.6138		0.2858	0.2704	↓↓
28.9	30.9	IPI00133839.1	Chaf1a Chromatin assembly factor 1 subunit A	2.466	0.863		0.6486	0.6138	↓↓
18.77	43.9	IPI00133985.1	Ruvbl1 RuvB-like 1	0.7943	0.912		0.2535	0.6026	↓↓
12.02	19	IPI00229432.8	Bptf Uncharacterized protein	0.9727	1.0765		0.5916	0.6607	↓↓
5.31	24.5	IPI00923656.1	Baz1b Isoform 1 of Tyrosine-protein kinase BAZ1B	0.896	0.6458		0.7516	0.3631	↓
3.74	14.3	IPI00556823.3	Prkaa1 5'-AMP-activated protein kinase catalytic subunit alpha-1	0.6918	0.6194	↓	1.1272	0.6982	

4.23	13.7	IPI00757755.1	Prkcb Isoform Beta-I of Protein kinase C beta type	4.3251	1.3305	↑	0.2466	1.4322	
7.89	28.4	IPI00131067.1	Mbd3 Isoform 1 of Methyl-CpG-binding domain protein 3	1.4454	0.9817		0.5702	0.6427	↓↓
proteins have structure roles in chromatin									
26.06	23.5	IPI00357096.3	Nipbl Isoform 2 of Nipped-B-like protein	2.0324	0.6138		0.1127	0.2582	↓↓
8.12	11.1	IPI00459898.3	Espl1 Separin	1.4322	1.1066		1.4588	1.7061	↑
18.33	29.6	IPI00135443.2	Top2b DNA topoisomerase 2-beta	0.302	0.7727		0.2312	0.52	↓↓
85.84	39.3	IPI00122223.1	Top2a DNA topoisomerase 2-alpha	0.3981	1.0093		0.0394	0.2355	↓↓
proteins involved in DNA replication									
5.62	11.5	IPI00125142.2	Msh3 DNA mismatch repair protein Msh3	1.3932	1.028		1.6749	1.556	↑↑
36.02	36.6	IPI00118158.1	Msh2 DNA mismatch repair protein Msh2	0.4169	0.4831	↓↓	0.5058	0.3499	↓↓
4.67	33.1	IPI00127014.1	Terf2ip Telomeric repeat-binding factor 2-interacting protein 1	1.977	0.3837		0.3698	0.1047	↓↓
22.79	53.5	IPI00648350.2	Trp53 cellular tumor antigen p53 isoform b	0.9908	0.9908		1.7378	1.3677	↑
proteins involved in DNA binding									
16.15	45.8	IPI00227720.3	Dek Protein DEK	0.5649	0.879		0.4018	0.1259	↓↓

4.85	19.5	IPI00314919.3	Supv3l1 ATP-dependent RNA helicase SUPV3L1, mitochondrial	1.2134	1.1169		0.7656	0.7516	↓
11.76	30.2	IPI00856664.1	Dnajc2 Uncharacterized protein	0.3192	0.6427	↓↓	0.1837	0.4786	↓↓
8	17	IPI00225459.1	Lrwd1 Leucine-rich repeat and WD repeat-containing protein 1	1.3677	1.6904	↑	0.2188	0.9727	
21.77	33	IPI00118853.1	Mre11a Isoform 1 of Double-strand break repair protein MRE11A	0.8395	0.8395		0.6252	0.6081	↓↓
2	11.5	IPI00830976.1	Nasp nuclear autoantigenic sperm protein isoform 2	0.7727	1.028		1.4454	1.4454	↑
proteins involved in other functions									
4	17.9	IPI00321739.1	Akap8 A-kinase anchor protein 8	0.929	1.0666		1.556	1.4454	↑
6.44	31.6	IPI00660262.4	Gpx4 phospholipid hydroperoxide glutathione peroxidase, nuclear isoform 1 precursor	0.871	0.7112		0.6546	0.4875	↓↓

5.2.2.2 Functional characterization of the proteins only regulated by 30 min or 4 h GnRH-induction

To further investigate the mechanisms which cause the LHβ and FSHβ to be differentially regulated by different GnRH pulses, we then analyzed the molecular functions of the proteins which were only regulated by 30 min or 4 h GnRH-treatment. Surprisingly, we found a few functions differentially presented among all the top-12 ranked molecular functions of each treatment. As shown in Figure 5.7, the function of calmodulin-binding was only found in 30 min GnRH-regulated proteins; in addition all the proteins identified with this function were up-regulated by 30 min GnRH-induction. While, the function of chromatin regulator was only found in 4 h GnRH-regulated proteins; and all the identified proteins involved in this function were down-regulated by 4 h GnRH-treatment. The differentially regulated proteins involve in these functions were listed in Table 5.4 and Table 5.5.

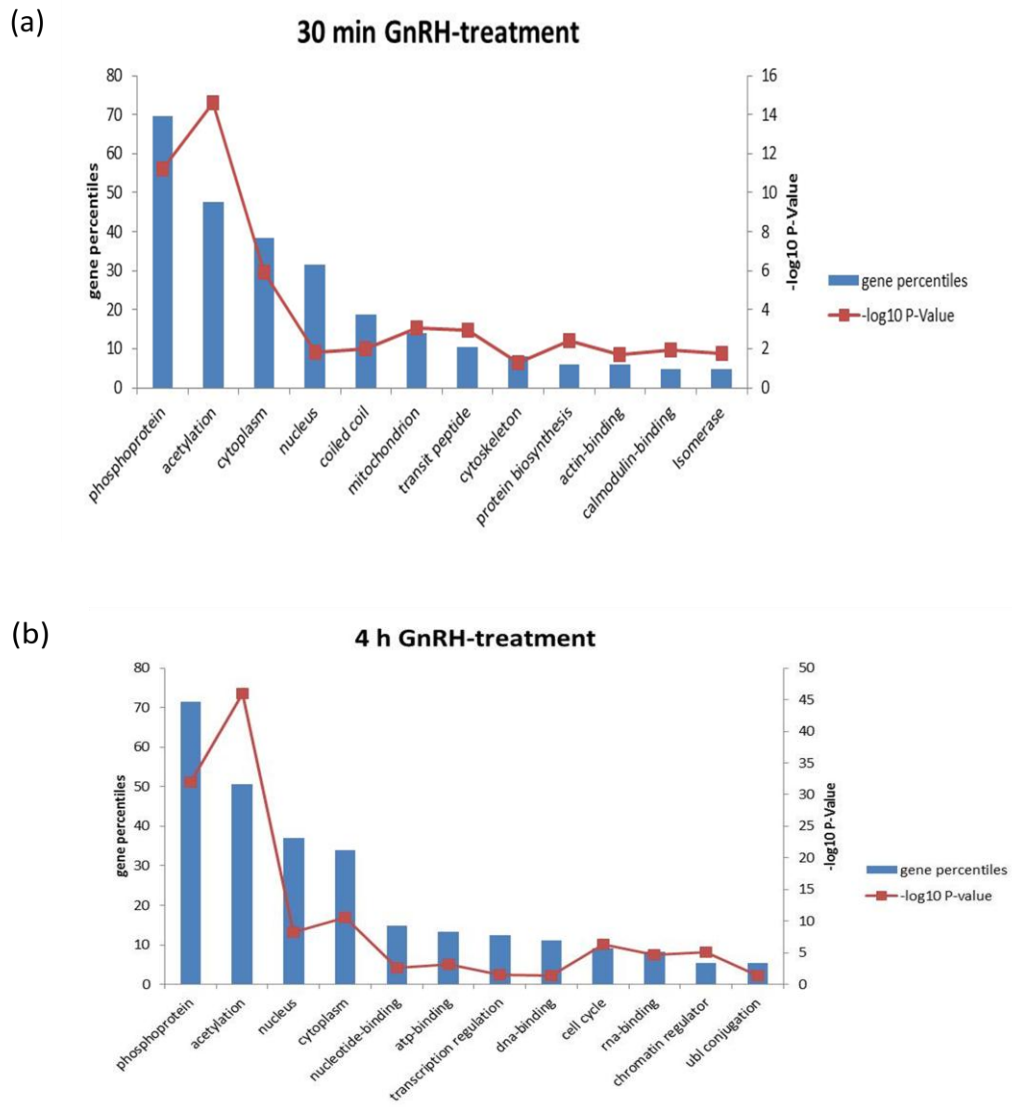


Figure 5.7 GO analysis of differentially regulated proteins by different GnRH frequency induction.

GO analysis was done using DAVID software. The percentiles of genes involved in each molecular function were plotted against the corresponding molecular function (bar). The Log10 P-value of each molecular function was also indicated in the graph (line). Only the molecular function with P <0.05 were shown in this graph. (a) The significantly altered proteins only regulated by 30 min GnRH-treatment (b) The significantly altered proteins only regulated by 4 h GnRH-treatment.

Table 5.4 Proteins involved in CaM-binding.

unused score	% seq cov	Accession #	Name	iTRAQ ratio (30 min GnRH)		Exp Pat.
				114:113	116:115	
21.39	38	IPI00387580.2	Add3 Isoform 1 of Gamma-adducin	4.1305	2.5823	↑
259.35	69.3	IPI00753793.2	Spna2 Isoform 2 of Spectrin alpha chain, brain	1.6444	3.4995	↑
2.21	27.9	IPI00119111.2	Cnn3 Calponin-3	1.5276	1.6444	↑
15.22	31.5	IPI00742310.5	Ewsr1 RNA-binding protein EWS	1.556	2.0137	↑

Table 5.5 Proteins involved in chromatin regulation.

unused score	% seq cov	Accession #	Name	iTRAQ ratio (4 h GnRH)		Exp Pattern
				118:117	121:119	
10.08	29.5	IPI00317722.3	Dmap1 Isoform 1 of DNA methyltransferase 1-associated protein 1	0.631	0.413	↓
10	28.7	IPI00396676.1	Suz12 Polycomb protein Suz12	0.2858	0.2704	↓
69.27	36.8	IPI00857777.1	Chd4 Uncharacterized protein	0.1941	0.2512	↓
18.77	43.9	IPI00133985.1	Ruvbl1 RuvB-like 1	0.2535	0.6026	↓
12	24.3	IPI00278600.1	Morf4l1 Isoform 1 of Mortality factor 4-like protein 1	0.52	0.6026	↓
8.02	17.9	IPI00762411.2	Whsc1 Isoform 1 of Probable histone-lysine N-methyltransferase NSD2	0.5495	0.3311	↓
15.64	25.5	IPI00875892.1	Kdm2a Uncharacterized protein (Fragment)	0.5105	0.3467	↓
13.95	22.7	IPI00274795.4	Ncor1 Uncharacterized protein	0.4742	0.4786	↓
16.65	37.5	IPI00649685.3	Smarcd2 Isoform 1 of SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 2	0.6252	0.6138	↓
31.65	43	IPI00122696.6	Rbbp4 Uncharacterized protein	0.3908	0.5105	↓
2.03	16.9	IPI00660988.3	Mll5 Isoform 1 of Histone-lysine N-methyltransferase MLL5	0.6546	0.5598	↓
100.52	52	IPI00469323.3	Dnmt1 DNA (cytosine-5)-methyltransferase 1 isoform 2	0.2655	0.597	↓

27.54	33.4	IPI00857769.1	Pbrm1 Uncharacterized protein	0.2965	0.3837	↓
4.72	67.7	IPI00338745.4	LOC100044391;Hmgn1 Non-histone chromosomal protein HMG-14	0.0871	0.2228	↓

Calmodulin (CaM) is a notable effector mediating GnRH-induced Ca^{2+} signaling downstream pathways through MAPK-independent manner (Melamed, Savulescu et al. 2012). Diverse studies have indicated the involvement of Ca^{2+} in the expression of gonadotropins (Naor 2009), and also suggested that Ca^{2+} sensing is a potential factor to decode GnRH pulse frequency (Haisenleder, Yasin et al. 1997). Therefore, our findings that the function of CaM binding is only presented in the proteins which were significantly regulated by 30 min supported the previous hypothesis. As the involvement of Ca^{2+} signaling in decoding the GnRH pulse frequency has been further suggested, we therefore used STRING to identify the significantly regulated proteins which were involved in Ca^{2+} signaling. These proteins were shown in Table 5.6.

Table 5.6 Proteins involved in Ca^{2+} signaling.

unused score	% seq cov	Accession #	Name	iTRAQ ratio (30 min treatment)			iTRAQ ratio (4 h treatment)		
				114:113	116:115	Exp Pat.	118:117	121:119	Exp Pat.
2.08	18.3	IPI00130419.1	Prkce Protein kinase C epsilon type	1.6444	1.5996	↑	1.1066	2.729	
4.23	13.7	IPI00757755.1	Prkcb Isoform Beta-I of Protein kinase C beta type	4.3251	1.3305	↑	0.2466	1.4322	
8.09	18.9	IPI00338964.3	Atp2a2 Isoform SERCA2B of Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	0.7586	0.4656	↓	0.5152	0.6026	↓
17.1	49.8	IPI00911151.1	Nucb2 nucleobindin-2 isoform 2	0.4487	0.6607	↓	1.3183	1.4191	↑
62.75	54.9	IPI00876528.1	Gtf2i Isoform 5 of General transcription factor II-I	0.9204	0.9204		0.6138	0.3908	↓
53.25	51.4	IPI00118899.1	Actn4 Alpha-actinin-4	1.2823	1.977	↑	0.4699	0.6668	↓

5.3 Discussion

5.3.1 Proteins involved in RNA processing

Alternative splicing (AS) is a unique process in higher eukaryote, which is driven by the spliceosome, a large ribonucleoprotein complex composed of small nuclear RNAs (snRNAs) and proteins (Fox-Walsh, Dou et al. 2005; Görnemann, Kotovic et al. 2005). It is regulated by positive and negative cis-elements such as exonic and intronic splicing silencers and enhancers (ESS, ISS, ESE, and ISE) as well as trans-acting factors such as heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine/arginine-rich (SR) proteins through a combinatorial interaction (Wu and Maniatis 1993). Besides, AS was also known to be regulated in a cell type- or developmental stage-specific manner, which were orchestrated by the amounts and activities of cellular splicing factors, such as RNA-binding motif (Rbm) proteins (Matlin, Clark et al. 2005). These proteins exert their functions through interacting with hnRNPs or SR proteins (Glisovic, Bachorik et al. 2008).

In our study, we found the spliceosome component proteins hnRNP h2 and Prpf 19 were differentially regulated by 30 min and 4 h GnRH, indicating different spliceosomes may be formed under different GnRH frequencies and therefore facilitating the AS of different gonadotropins. We also identified a number of Rbm proteins and most of them were regulated by 4 h GnRH-treatment. These proteins have been implicated in other context to modulate the pre-mRNA processing through their functional motifs (Zhou, Ou et al. 2008; Lin and Tarn 2011; Niu, Jin et al. 2012). So identification of the interacting partners of these Rbms will be needed to elucidate how they regulate pre-mRNA processing in the events of gonadotropin regulation. We also found a number of SR

proteins were regulated by 30 min GnRH-treatment. SR proteins are essential factors participating in the maturation of the spliceosome. These proteins have a C-terminal domain rich in Arg-Ser repeats (RS domain) and one or two N-terminal RNA recognition motifs (RRMs). RRMs modulate SR protein interactions in the spliceosome by binding short mRNA sequences, and the phosphorylation of RS domain is required for translocation of SR proteins into the nucleus, and the translocation was suggested to be crucial for alternative splicing, mRNA export, and other RNA processing events (Ghosh and Adams 2011). We found that the SR proteins Sfrs3 was up-regulated and Sfrs5 was down-regulated by 30 min GnRH-treatment in LβT2 nuclear fraction, indicating translocation may happen, and most likely be triggered by GnRH-induced phosphorylation. SR protein can be phosphorylated by two protein kinase families, one of which is the family of SRPKs. These kinases directly affect nuclear import of SR proteins (Gui, Lane et al. 1994). Furthermore, phosphorylation induced by GnRH were mainly through MAPK pathways, and a previous study identified a small insert, called MAPK insert, located in SPPK1 kinase domain (Zhou, Qiu et al. 2012). So we proposed that GnRH might cause phosphorylation and subsequent translocation of SR proteins through MAPK pathways, and result in the regulation of gonadotropin mRNA splicing and maturation. The involvement of GnRH-regulated proteins in RNA processing was summarized in Figure 5.8. Further works are needed to prove this hypothesis.

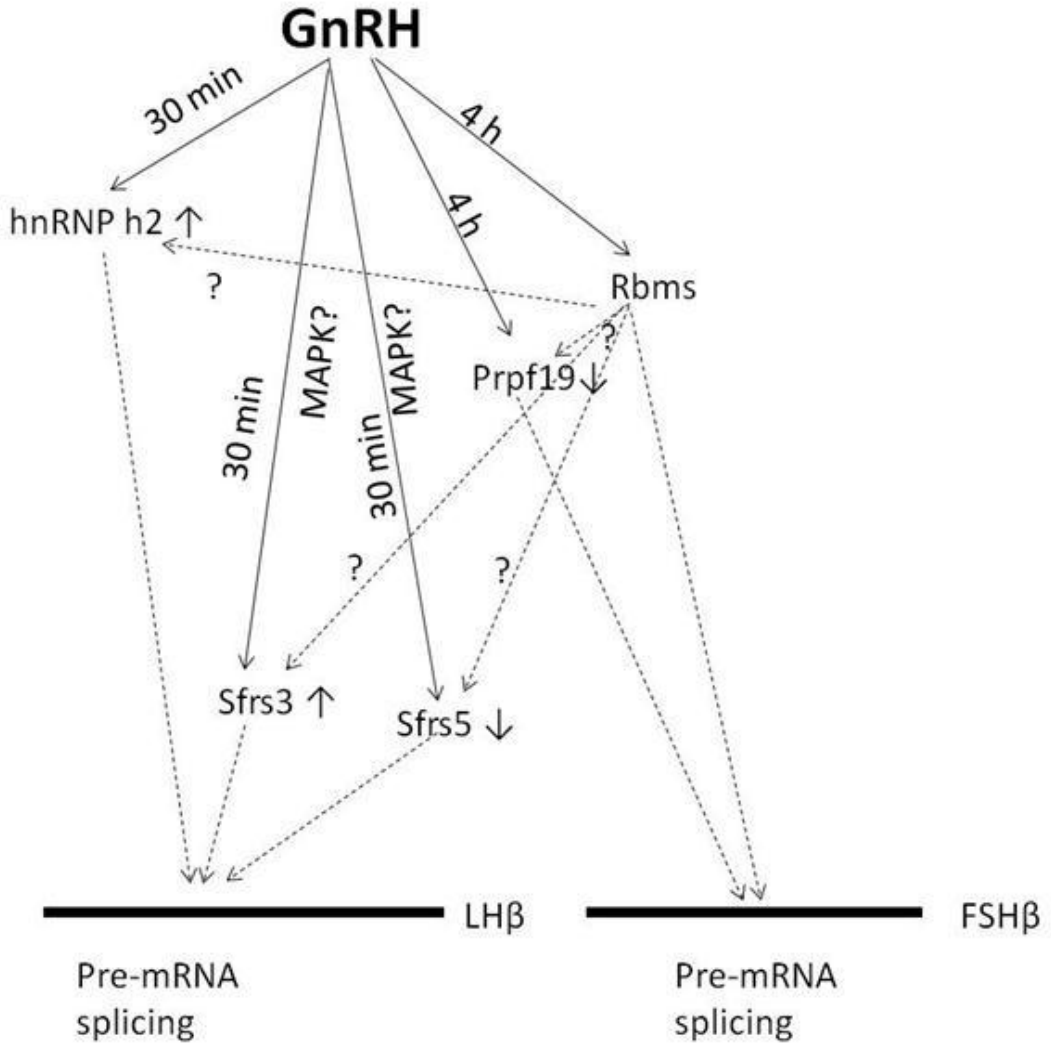


Figure 5.8 The involvement of GnRH-regulated proteins in RNA processing.

GnRH pulse differentially regulates the trans-acting factors of AS process, including hnRNP h2, Prpf19, and SR proteins. These factors may consequently mediate the GnRH signals to regulate the pre-mRNA splicing of gonadotropins. Beside, GnRH also regulates a number of Rbms. These Rbms may interact with hnRNP h2, or Prpf19, or SR proteins, or with other splicing factors and eventually acts on the AS process. (solid arrows indicate the results derived from literature, and dashed arrows indicate the speculated results; ↑ stands for up-regulation, ↓stands for down-regulation)

5.3.2 Proteins involved in translation

Eukaryotic cap-dependent translation initiation involves a process to form an elongation-competent 80S ribosome. This process requires the cooperation of at least nine eukaryotic initiation factors (eIFs) and comprises two steps: the formation of 48S initiation complexes and the joining of 48S complexes with 60S subunits (Jackson, Hellen et al. 2010). In our study, we identified several eukaryotic translation initiators, whose expression levels were altered by GnRH, indicating the impact of GnRH on the process of translation initiation. Our study found that Eif5 was down-regulated by both 30 min and 4 h GnRH-treatments. The function of eIF5 was implicated in the final step of AUG selection- release of Met-tRNAi from eIF2-GDP (Sokabe, Fraser et al. 2012). Moreover, eIF5 was a critical component of eIF2(αP) regulatory complex, which modulated phosphorylation of eIF2, restricting recycling of eIF2 in the process of translation initiation (Jennings and Pavitt 2010). Therefore, our finding is consistent with the expectation that reduced amount of Eif5 will contribute to less inhibitory effect on eIF2 circulation, thus facilitating the process of translation initiation.

Besides, eIF4E was up-regulated by 4 h GnRH-treatment while without any significant changes by 30 min GnRH-treatment. The phosphorylation of eIF4E has been implicated to be sensitive to GnRH treatment in LβT2 cells, and was found through ERK activation (Nguyen, Santos et al. 2004). So how the phosphorylation status affect the association between eIF4E and different translation initiation complexes would be an interesting subject to explore. eIF3 is a large complex composed of 13 subunits (a-m) in mammals, playing a crucial role in recruiting mRNA to 43S ribosome (Jackson, Hellen et al. 2010). We found Eif3l was down-regulated upon 30 min GnRH-treatment, while slightly up-

regulated (not significant for both replicates) by 4 h GnRH treatment, implicated that the translation initiation factors may differentially act on the translation machinery driven by GnRH pulse variance.

We also identified a number of aminoacyl-tRNA synthetases (aa-RSs), which are regulated by GnRH treatment. aa-RSs are crucial regulators for faithful translation from mRNA to protein, a process involving two steps: forming the aminoacyl-tRNAs (aa-tRNAs) by aa-RSs and delivering them to the ribosome by elongation factors (Ogle and Ramakrishnan 2005). The specificity of the amino acid substrates is achieved by means of preferential binding of the cognate amino acids and selective editing of near-cognate amino acids. So far there are two types of intrinsic proofreading activities, either before (pre-transfer editing) or after (post-transfer editing) the mis-activated amino acid is attached to tRNA, have been reported to be carried out by aa-RSs (Ling, Reynolds et al. 2009). In this study, we found GnRH treatment caused the differential regulation of aa-RSs, which have never been reported by other groups before. Further validation will be needed to identify the specific targets of the aa-RSs. The involvement of GnRH-regulated proteins in translation was summarized in Figure 5.9.

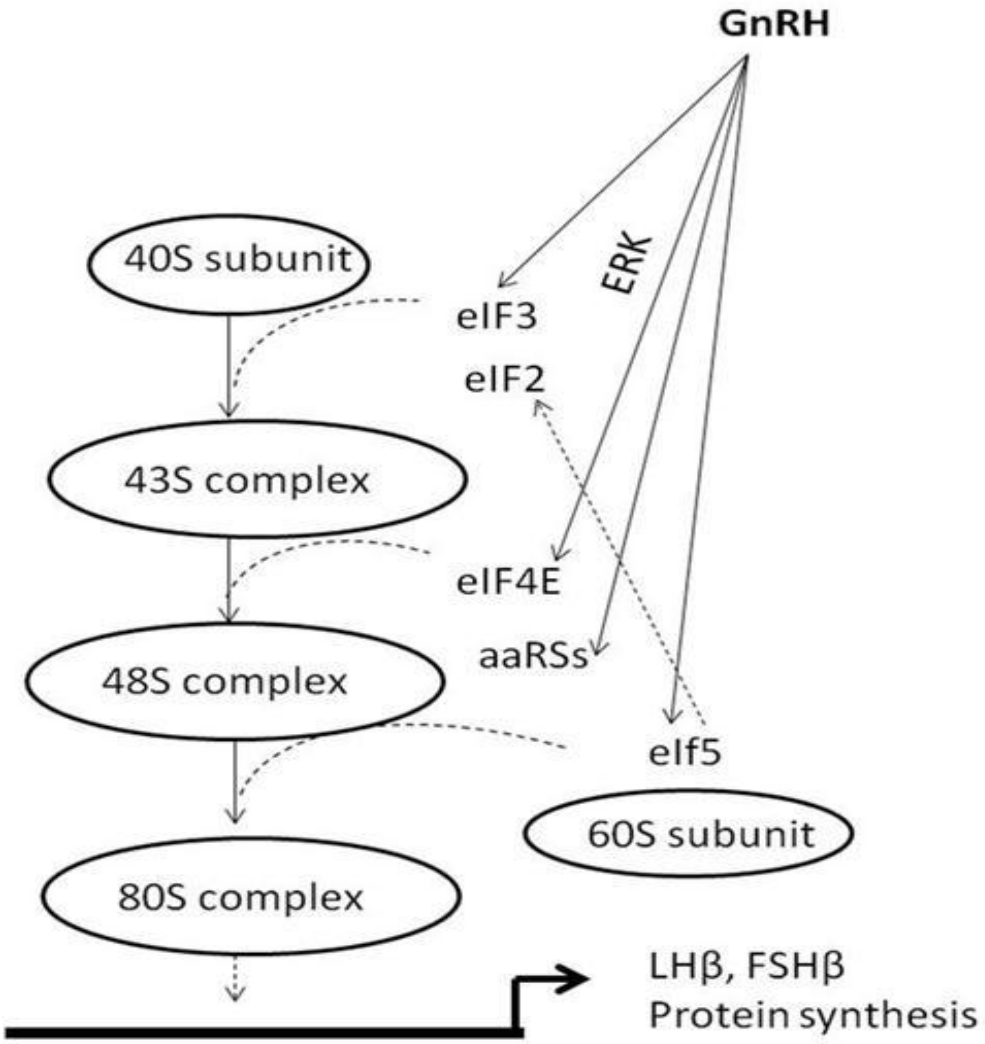


Figure 5.9 The involvement of GnRH-regulated proteins in translation.

GnRH regulates several translational initiation factors, which may consequently affect the assembling of translation initiation machinery from corresponding steps. GnRH also regulates the aaRSs, which may subsequently affect the process of aa-tRNA generation. The effect on translational initiation factors and aaRSs, will function together on the regulation of gonadotropin translation.

5.3.3 Proteins involved in chromosome organization

In eukaryotic cells, DNA is packed in the nucleus to form a highly organized form of chromosome. Nucleosome is the basic unit of chromatin. It is made of 145-147 bps of DNA which is wrapped around an octamer consisting of two of each of the histones H2A, H2B, H3 and H4. Nucleosome is repeated and chromatin is further condensed throughout the genome. It has been suggested that the specific composition of chromatin is an important determinant of the cellular function (Kouzarides 2007). PTMs of the N-terminal tails of each core histone, which modulate the chromatin structure, play crucial roles in the regulation of cellular processes. Such PTMs include acetylation, methylation, phosphorylation, ubiquitylation and SUMOylation (Kooistra and Helin 2012). Among all the PTMs, histone deacetylation has been recently studied in the gonadotrope cells (Lim, Luo et al. 2007; Melamed 2008). It was found that the HDACs caused repression of gonadotropin gene expression can be overcomed by GnRH signaling. In our study, we found a number of proteins which were predicted to interact with HDACs. NcoR1 uncharacterized protein was down-regulated by GnRH-treatment. NcoR is one of the well-known corepressors that lack enzymatic activity but can recruit HDACs to the specific gene loci or to stabilize the HDAC complex (Glass and Rosenfeld 2000; Jepsen and Rosenfeld 2002). The N-terminus of NcoR is composed of three repression domains, which allow interaction with several HDACs and also with mSin3A, another common corepressor. Sin3 can directly interact with HDACs and DNA-bound factors through its multi-domain to form a scaffold protein (Glass and Rosenfeld 2000; Grozinger and Schreiber 2002). So it is possible that decreased amount of NCoR1 by GnRH in nucleus will result in less HDACs recruited to the gonadotropin promoters. Further work will be

done to find the specific HDACs associated with NcoR1 and also the specific promoter targeted by the HDAC repressor complex.

Chd4 uncharacterized protein was also down-regulated by GnRH-treatment. Chd4 is the core subunit of a chromatin remodeling complex, the Nucleosome Remodeling and Deacetylase (NuRD) complex, which plays important roles in repressing transcription in many context of biological processes (Xue, Wong et al. 1998; Pegoraro, Kubben et al. 2009). Chd4 is composed of three crucial functional domains, a SNF2-type ATPase domain, a tandem plant homeodomain (tPHD), and a tandem chromodomain (tCHD). tPHD domain is found in many chromatin remodeling factors and has been suggested to be involved in nucleosome/histone binding (Bienz 2006; Peña, Davrazou et al. 2006; Shi, Kachirskaia et al. 2007). tCHD domain was shown to mediate chromatin interaction by directly binding with DNA, RNA or methylated histone H3 (Akhtar, Zink et al. 2000; Bouazoune, Mitterweger et al. 2002; Flanagan, Mi et al. 2005; Flanagan, Blus et al. 2007). NuRD is a transcription repressor. it has been reported to regulate a diversity of biological processes through forming multicomplex with Chd4 and Chd4-associated subunits, such as with HDAC1 and HDAC2 (Bowen, Fujita et al. 2004). Therefore, Chd4 has a great potential to regulate the transcription of gonadotropins through mediating the formation of repressor complex with regulatory proteins or HDACs. Future work will be carried out to identify these Chd4-associated factors.

Hmgn1 was found to be differentially regulated by 30 min and 4 h GnRH-treatment. This protein is able to bind specifically to nucleosomes (Bustin and Reeves 1996), especially on the regulatory region (Cuddapah, Schones et al. 2011). The presence of this protein on the chromatin and nucleosomes thereby facilitates the accessibility of other regulatory

factors to their targets. The accessibility of a molecule to the chromatin is quite dependent on its cellular localization. The nuclear Hmgn1 level is higher upon 30 min than 4 h GnRH-treatment thus indicating different GnRH pulse frequency may affect the nucleus translocation of Hmgn1, and Hmgn1 may have more chance to associate with nucleosome under 30 min treatment. Except for the cellular localization, the PTMs of Hmgn1, mainly through acetylation and phosphorylation, were also suggested to be crucial for modulating the activity of Hmgn1 (Pogna, Clayton et al. 2010). So whether different GnRH pulses also regulate the modification of Hmgn1is also worth to be investigated. We believe the correlation between the GnRH pulse and the cellular localization as well as modification of Hmgn1 may help us to understand the mechanism of GnRH pulse frequency decoding in terms of chromatin remodeling. The involvement of GnRH-regulated proteins in chromosome organization was summarized in Figure 5.10.

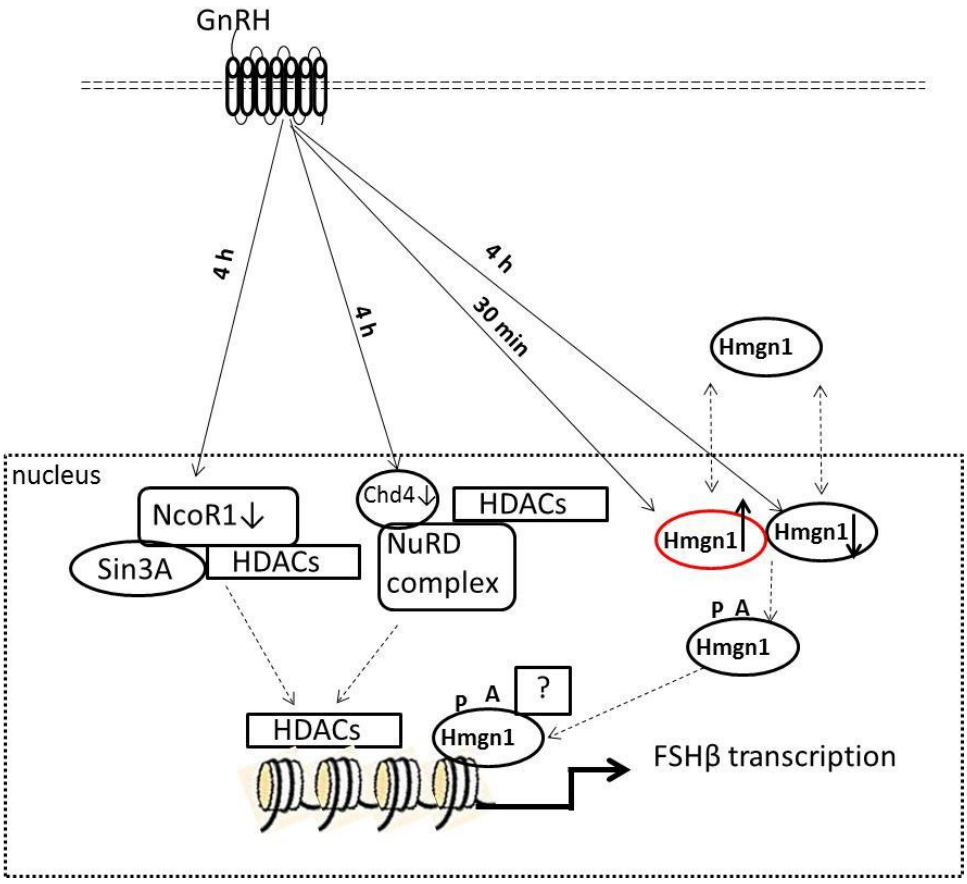


Figure 5.10 The involvement of GnRH-regulated proteins in chromosome organization.

GnRH down-regulates NcoR and Chd4. NcoR is a chromosome co-repressor, which works together with Sin3A to recruit HDACs to the chromosome and inhibit the transcription of the target chromosome. Chd4 is the subunit of NuRD complex, which also recruits HDACs to the chromosome and inhibits the transcription of the target chromosome. So down-regulation of NcoR and Chd4 by GnRH may inhibit the repression on the gonadotropin genes. Hmgn1 is differentially regulated by 30 min and 4 h GnRH-treatment. The nucleus translocation and modification of Hmgn1 were possibly affected. So far all the known factors involved in chromosome remodeling targeted FSHβ gene transcription. (solid arrows indicate the results derived from literature, and dashed arrows indicate the speculated results; ↑ stands for up-regulation, ↓stands for down-regulation)

5.3.4 Ca^{2+} signaling involved in decoding the GnRH pulse frequency

In this study we identified a number of proteins involved in regulating Ca^{2+} signaling are differentially regulated by GnRH pulse frequency. Ca^{2+} signaling has been suggested to be involved in decoding the GnRH frequency driven by the intermittent changes in Ca^{2+} concentration (Haisenleder, Yasin et al. 1997). Although external calcium influx and calcium mobilization from internal IP_3-sensitive stores (Naor, Capponi et al. 1988; Stojilković, Iida et al. 1991; STOJILKOVIC, REINHART et al. 1994) have been suggested to be the causes of intermittent changes in Ca^{2+} concentration, regulators of G-protein signaling (RGS) proteins, which serve as GTPase-activating proteins for Gαi, Gαq, and Gα12 subunits (Ross and Wilkie 2000), have been implicated in mediating GnRH-induced Ca^{2+} signaling (Naor, Capponi et al. 1988). However, it is not yet clear how GnRH regulates these processes.

In our study, Gtf2i was found to be down-regulated by 4 h GnRH-treatment, but not by 30 min GnRH-treatment. Gtf2i was reported to negatively regulate the transient receptor potential channel 3 (TRPC3), a Ca^{2+} channel, through binding with phospholipase C (PLC) and inhibit the entry of Ca^{2+} (Park and Dolmetsch 2006). So it is possible that 4 h GnRH-treatment decreases the level of Gtf2i, and consequently allows the PLC binding with TRPC3 instead of Gtf2i, therefore facilitating the activation of TRPC3 calcium channel, and results in increased Ca^{2+} influx. On the contrary, 30 min GnRH-treatment does not affect the level of Gtf2i therefore won't affect the binding of TRPC3 with PLC and the consequent effects. Nucb2 was another potential Ca^{2+} signaling regulator. Nucb2 was down-regulated by 30 min GnRH-treatment while up-regulated by 4 h GnRH-treatment. Nucb2 was found to have a common motif with Gα-interacting vesicles-

associated protein (GIV), which is the first non-receptor guanine nucleotide exchange factor (GEF) that activates Gαi (Garcia-Marcos, Kietrsunthorn et al. 2011). Increased level of Nucb2, which was induced by 4 h GnRH-treatment, may activate Gαi and downstream PLCβ, which may lead to the increasing of Ca^{2+} concentration. On the contrary, decreasing Nucb2 by 30 min GnRH-treatment may conversely regulate Ca^{2+} concentration. Except for regulating Ca^{2+} concentration, we also identified the proteins which may play roles in mediating Ca^{2+} signaling to gonadotropin genes. We identified a group of CaM-binding proteins which were only regulated by 30 min treatment. As CaM is a downstream effector of GnRH-induced Ca^{2+} signaling, these CaM-binding proteins may function as CaM-downstream effectors to gonadotropins through CaMKs pathway or calcineurin pathway (Melamed, Savulescu et al. 2012), or other novel pathways. We also identified a protein, ACTN4, which was differentially regulated by different GnRH pulse frequency. ACTN4 has a CaM-like domain, so it might mimic the function of CaM and regulate the gonadotropins. Hence future work will be done to validate the functions of these proteins. The involvement of the proteins in Ca^{2+} signaling was summarized in

Figure 5.11.

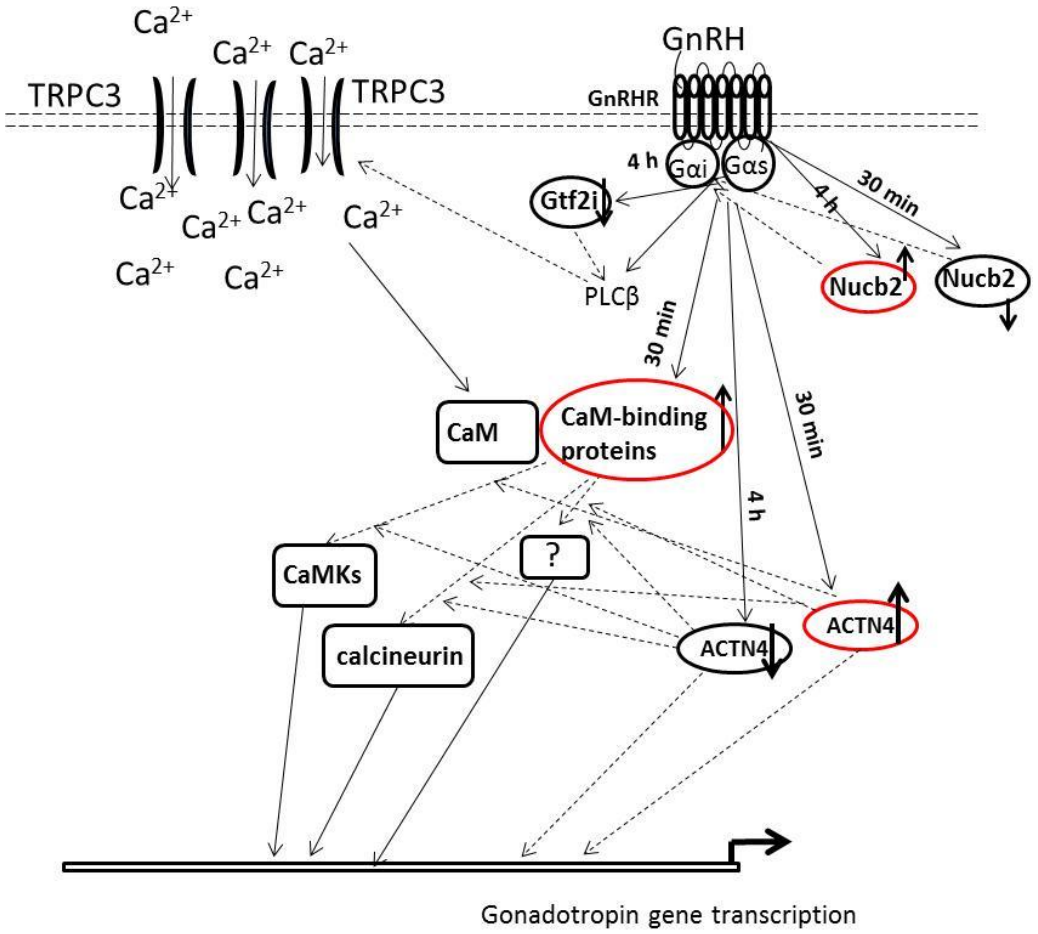


Figure 5.11 The involvement of GnRH-regulated proteins in Ca^{2+} signaling.

Gtf2i was differentially regulated by GnRH pulse frequency and consequently affect the binding of PLC and TRPC3, which eventually results in varied Ca^{2+} influx. Nucb2 was differentially regulated by GnRH pulse frequency, and differentially acts on Gαi and downstream PLCβ, which may lead to the varied Ca^{2+} concentration. A group of CaM-binding proteins were only regulated by 30 min GnRH treatment. They may act on the gonadotropins through CaMKs pathway or calcineurin pathway or other novel pathways. ACTN4 was differentially regulated by different GnRH pulse frequencies. Its CaM-like domain may function similar as CaM, which affects CaMKs pathway or calcineurin pathway or other novel pathways, results in the regulation of gonadotropins. (solid arrows indicate the results derived from the literature, and dashed arrows indicate the speculated results; ↑ stands for up-regulation, ↓ stands for down-regulation.)

In conclusion, our study provides preliminary evidence showing the novel regulatory role of GnRH in the regulation of gonadotropins. However, we are not able to specify the function of each molecule in term of the regulation. Further work will be needed to identify the targets which mediate the effect to the gonadotropin. Moreover, we found crucial factors, which are involved in regulating calcium concentration, CaM-binding and CaM downstream signaling, were differentially regulated by GnRH pulse frequency. These findings further support the previous hypothesis that Ca^{2+} signaling is crucial in decoding the GnRH frequency. The proposed functional roles of these potential regulators, however, need to be validated. Our iTARQ study provided valuable clues and suggestions for understanding the multifunctional roles of GnRH in the regulation of gonadotropins and building the interactive GnRH signaling pathways.

Chapter 6. Alpha-actinin 4-nuclear translocation mediates gonadotropin-releasing hormone stimulation of follicle-stimulating hormone β-subunit protein expression in LβT2 cells

6.1 Introduction

In last chapter, by using the iTRAQ-based proteomic profiling, we identified a number of proteins which were involved in Ca^{2+} signaling. Ca^{2+} signaling has been suggested to be involved in decoding the GnRH frequency driven by the intermittent changes in Ca^{2+} concentration (Haisenleder, Yasin et al. 1997). So we expect that these regulated proteins may function differentially on *Lhβ* and *Fshβ* genes. One of them is actinin 4 (ACTN4). It carries a C-terminal calmodulin (CaM)-like domain, which has the potential to exert a similar function as CaM protein, the downstream effector of GnRH-induced Ca^{2+} signaling, on the gonadotropin gene. To validate the function of ACTN4, we carried out the following study.

Our study proved that ACTN4 is up-regulated in the nucleus of LβT2 cells induced by GnRH. Functional studies showed that ACTN4 may be a potential positive regulator of *Fshβ* gene transcription, likely through its C-terminal CaM-like domain.

6.2 Results

6.2.1 GnRH induces nuclear translocation of ACTN4

ACTN4 was one of the proteins identified by the iTRAQ experiment, which showed significant abundance change upon 30 min GnRH-treatment. To confirm the up-regulation of ACTN4 in the nucleus of GnRH-treated LβT2 cells, we examined the protein levels of ACTN4 in nucleus and cytoplasm by western blot analysis (Figure 6.1). The result demonstrates that GnRH stimulation increased the protein level of nuclear ACTN4 in both biological replicates (1.69±0.21-fold and 1.82±0.07-fold, respectively), which was consistent with our iTRAQ result.

Immunofluorescence confocal microscope study was performed to detect the subcellular distribution of endogenous ACTN4 in LβT2 cells after GnRH induction. In the mock-treated cells, ACTN4 was predominantly accumulated as a circle surrounding the inner layer of plasma membrane. This is consistent with its role as a cytoskeleton protein. After GnRH treatment significant amount of ACTN4 was redistributed to nucleus as indicated in Figure 6.2 (a). Quantitation data showed that nuclear ACTN4 level increased by 1.93±0.25 fold, confirming nuclear translocation of ACTN4 induced by GnRH (Figure 6.2 (b)).

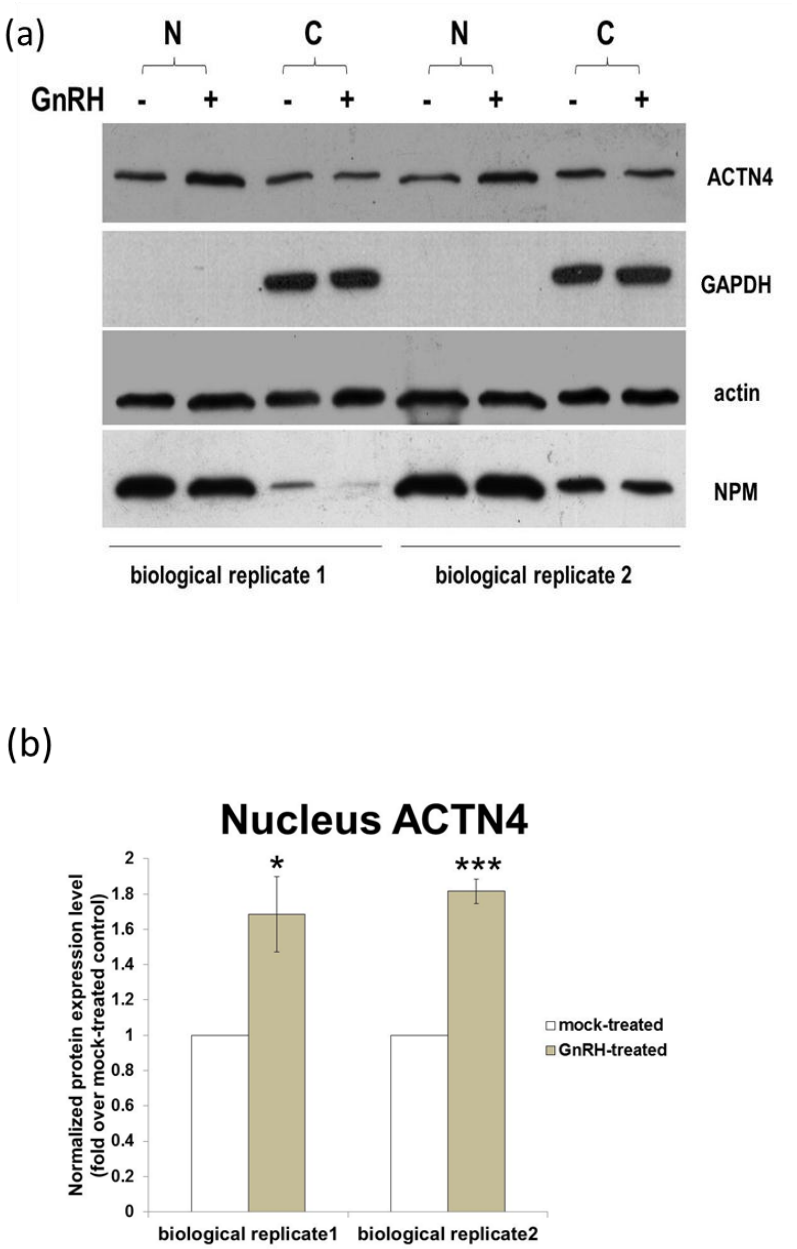


Figure 6.1 Western blot validation of iTRAQ experiment.

LβT2 cells from each of the two biological replicates were treated with 100 nM GnRH or mock. The nuclear (N) and cytoplasmic (C) fractions were separated as indicated in Materials and Methods. (a) The expression level of ACTN4 in each fraction was measured by western blot. NPM and GAPDH were the loading controls for nuclear and cytoplasm fractions, respectively. Representative blot was shown. (b) Quantification of nuclear ACTN4 was performed from three independent experiments (NPM was set as the control for normalization of nuclear protein) and the mean fold change over control ±SEM was presented. *P<0.05, ***P<0.001.

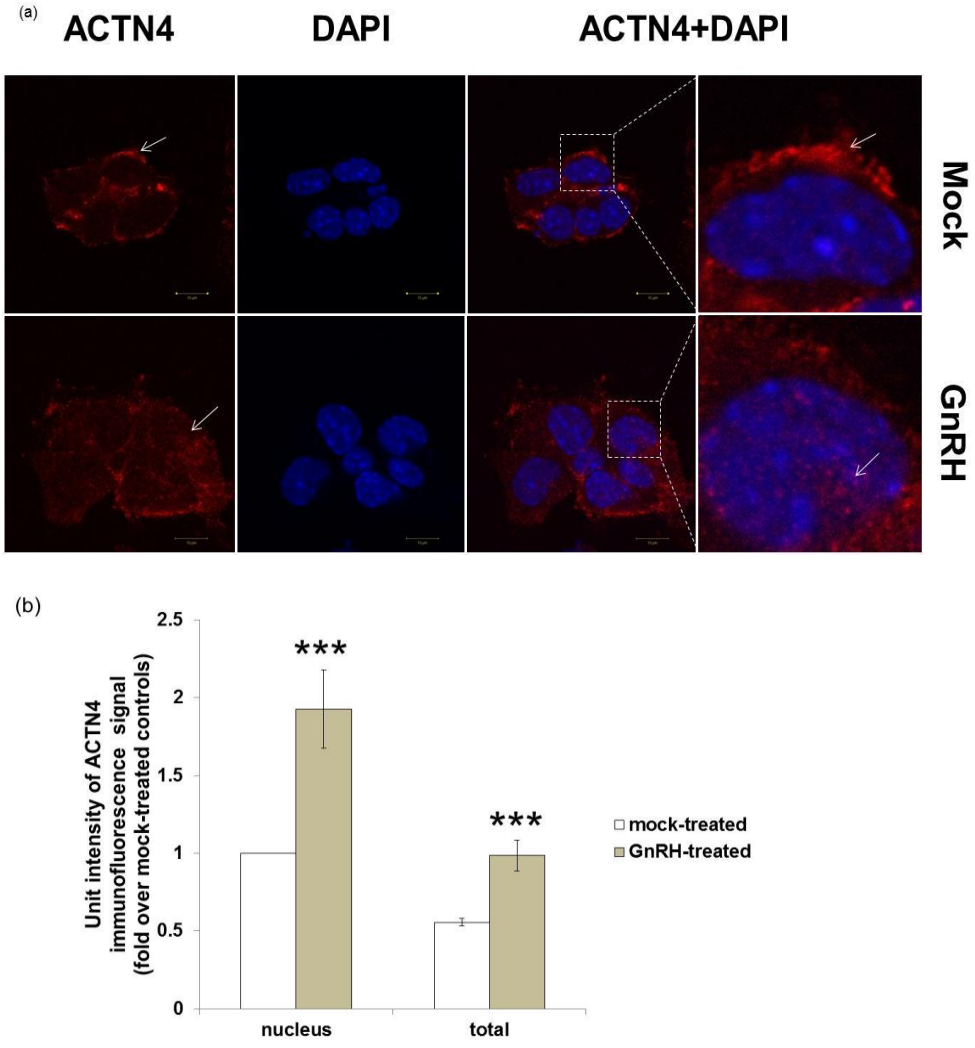


Figure 6.2 Immunofluorescence analysis for intracellular distribution of ACTN4 after GnRH-induction in LβT2 cells.

(a) Redistribution of ACTN4 after GnRH treatment is indicated by the white arrows. The zoom-in images of single cells (marked by the white squares) from both mock-treated and GnRH-treated groups were shown at the right side of the panels. LβT2 cells were seeded on pre-coated coverslips and serum-starved for 12 hrs, and were exposed to 100 nM GnRH or mock. Cells were fixed and then stained for ACTN4 (red) and nuclei with DAPI (blue). Scale bar=10 μm. (b) Quantification of confocal images. Results were expressed as fold change normalized to those of mock-treated controls. Values are mean±SEM (***P<0.001).

ACTN4 is originally recognized as an actin-binding protein and predominantly localized at stress fibers, microfilament bundles, and sites of cell contact or adhesion, engaged in organization of actin cytoskeleton and regulation of cell motility (Honda, Yamada et al. 1998). The translocation of ACTN4 from adhension site to nucleus through actin fibers upon hormonal stimulation is an efficient signaling pathway. Besides, the translocation of ACTN4 may result in the actin reorganization and cause the change of the cell morphology and activate the gene transcription. It was only until recently that the nuclear functions of ACTN4, as a transcription factor, started to be unveiled (Poch, Al-Kassim et al. 2004; Chakraborty, Reineke et al. 2006; Babakov, Petukhova et al. 2008; Khotin, Turoverova et al. 2010; Khurana, Chakraborty et al. 2011).

6.2.2 Effects of ACTN4 on the transcription of mouse gonadotropin β-subunit genes and GnRHR gene

As we found that ACTN4 nuclear translocation is increased upon GnRH treatment, we speculated that ACTN4 may also play a role in regulating transcription of gonadotropins. To test this hypothesis, the effects of overexpression and knock-down of ACTN4 were studied. Figure 6.3 is the western blot confirmation showing the effective up-regulation (7-fold) of ACTN4 by ACTN4 OE and down-regulation (4-fold) by siACTN42032. Since the knock-down efficiency of siACTN4976 was not as significant as that of siACTN42032, we only used siACTN42032 for the following experiments. We examined the effect of ACTN4 on the transcription of three of the tertiary gonadotrope signature genes (Salisbury, Binder et al. 2008): Fshβ, Gnrhr and Lhβ. RT-PCR result demonstrated that transient transfection of ACTN4 OE into LβT2 cells markedly promoted mFshβ mRNA level, while did not affect transcript levels of mLhβ and mGnrhr (Figure 6.4). The effect on mFshβ mRNA was further verified by quantitative real-time PCR (Figure 6.5).

ACTN4 OE increased the mRNA level of mFshβ gene to 1.46±0.13 fold ($P<0.05$), while siACTN42032 reduced mRNA level of mFshβ gene to 0.69±0.17 fold ($P=0.07$). The effect of siACTN42032 on the transcript level of mFshβ gene, though not statistically significant, showed opposite trend to the effect by ACTN4 OE. These findings indicate that ACTN4 positively regulates the transcription of mFshβ gene.

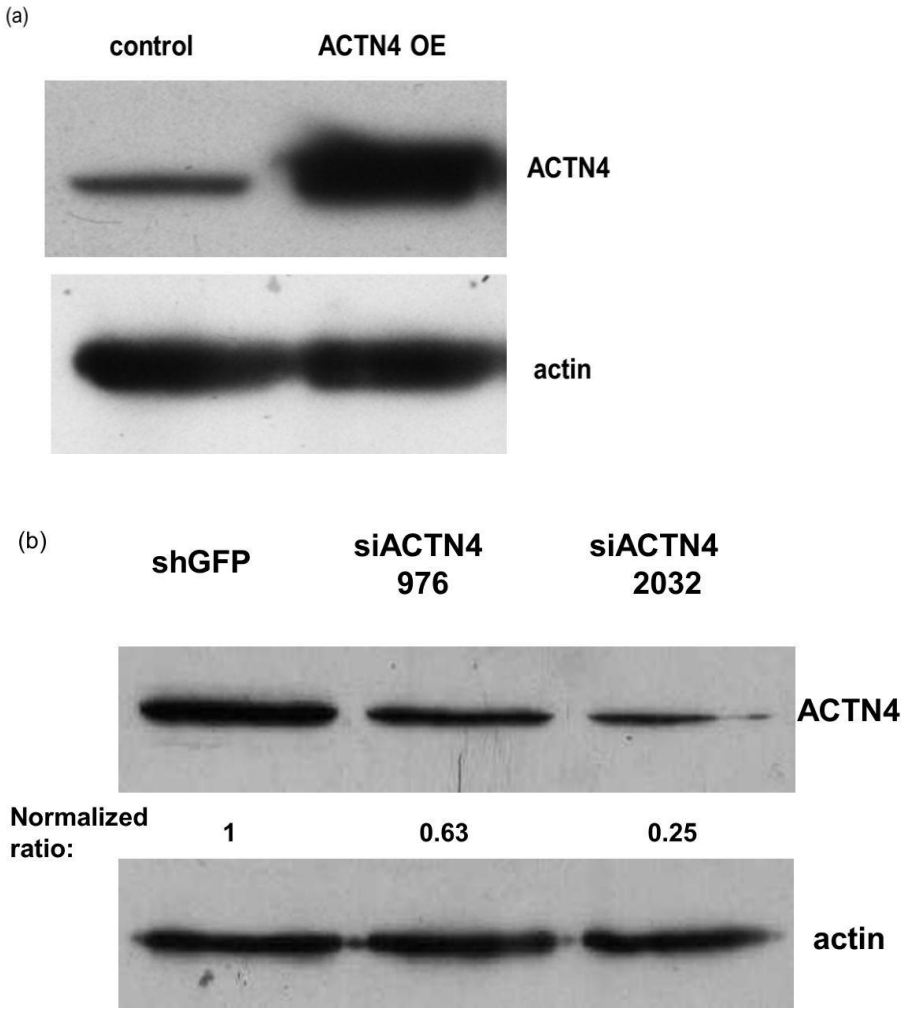


Figure 6.3 Confirmation of the ACTN4 OE and knocking-down constructs by western blot.

The expression level of ACTN4 OE (a) and knocking down (b) were assessed by western blot with actin as a loading control.

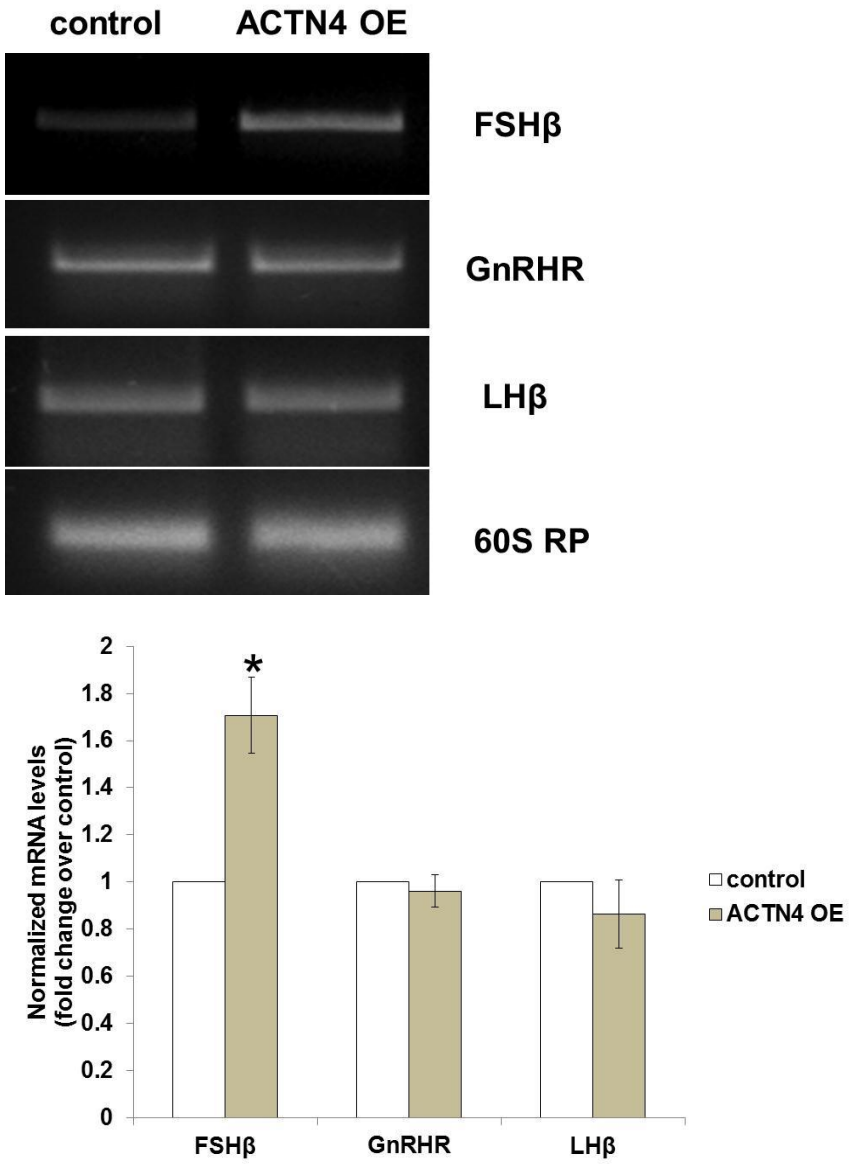


Figure 6.4 RT-PCR of ACTN4 effects on *mFshβ*, *mGnrhr* and *mLhβ* genes.

pXJ40-FLAG-ACTN4 was transfected into LβT2 cells, total RNA was extracted. *mFshβ*, *mGnrhr* and *mLhβ* mRNA levels were measured by RT-PCR using 60S RP as an internal control, and levels are showed as fold change over those in pXJ40-FLAG transfected LβT2 cells. Values are mean±SEM of three independent experiments. *t* test comparing means between groups with or without ACTN4 over-expression were conducted for each group. *P<0.05.

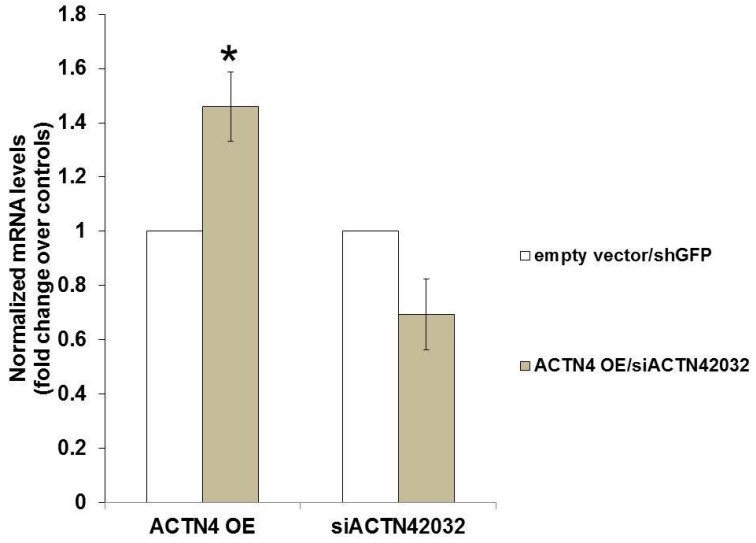


Figure 6.5 Quantitative real-time PCR of *mFshβ* gene.

The experiment was performed using primers spanning an intron/exon border of the *mFshβ* gene. Changes in cycle threshold values were normalized to those of 60S RP and compared to the levels in the cells transfected with control plasmids. Values are mean±SEM of three independent experiments. *P<0.05.

6.2.3 ACTN4 regulates mFshβ promoter

Since the amount of ACTN4 in nucleus increases after GnRH treatment, we speculated that ACTN4 may exert its regulatory roles on the *mFshβ* promoter. Hence, we performed luciferase promoter assays to assess the effect of ACTN4 on *mFshβ* promoter activity. As shown in Figure 6.6, transient transfection of ACTN4 OE dramatically increased *mFshβ* promoter activity by 1.86±0.06 fold ($P<0.001$), while $siACTN4_{2032}$ repressed this promoter activity to 0.59±0.04 fold ($P<0.001$). Collectively, our data suggest that ACTN4 is a positive regulator of *mFshβ* gene transcription, and likely has functional impact on *mFshβ* promoter.

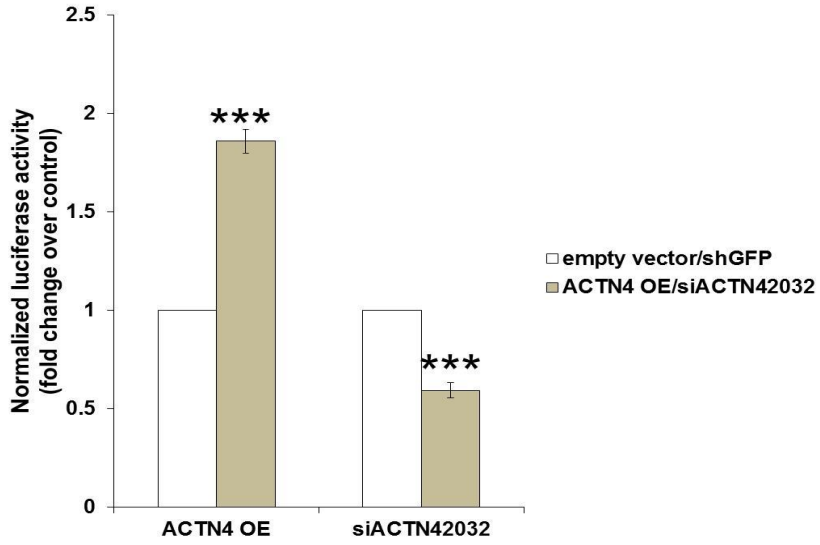


Figure 6.6 Effects of ACTN4 on mFshβ promoter.

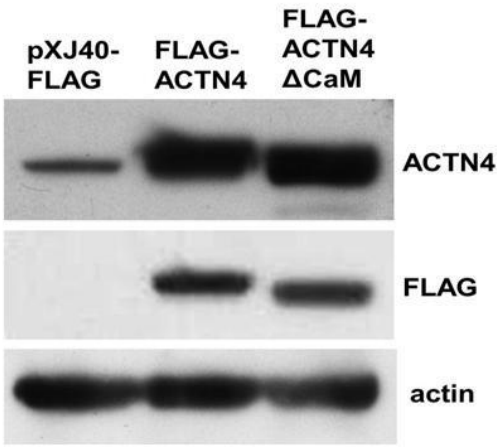
mFshβ promoter-firefly luciferase constructs were transiently transfected into LβT2 cells together with ACTN4 OE/empty vector or siACTN4/shGFP. Firefly luciferase values were normalized to those of Renilla luciferase, and were shown as fold changes over levels in vector-transfected LβT2 cells. Values are mean±SEM of two independent experiments, each performed in four replicates. ***P<0.001.

6.2.4 ACTN4-induced transcriptional activation of mFshβ requires the CaM-like domain

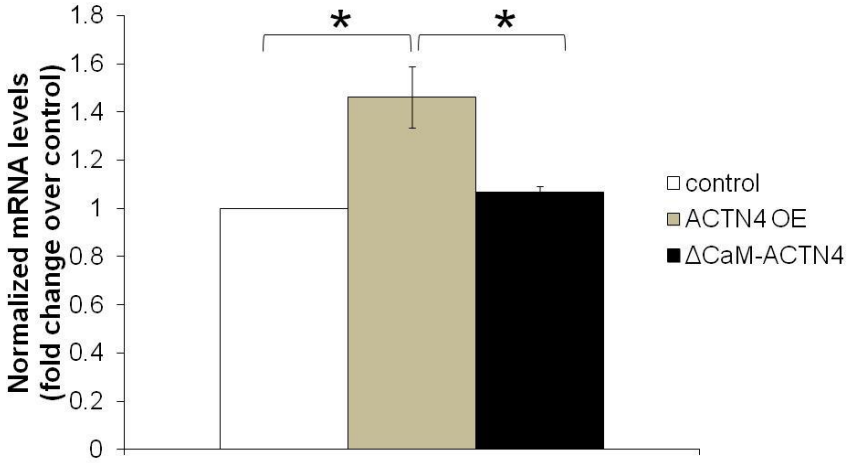
After verifying the effect of ACTN4 on the transcription of *mFshβ* gene, we further investigated which domain of ACTN4 plays a role in the transcriptional regulation. ACTN4 contains an N-terminal actin-binding domain, a central four spectrin-repeat domain, two EF-hand Ca^{2+}-binding domains and a C-terminal CaM-like domain. Previous studies have found that the CaM-like domain of ACTN4 can antagonize histone deacetylase 7 (HDAC7) and consequently potentiate myocyte enhancer factor-2 (MEF2) transcription activity (Chakraborty, Reineke et al. 2006). Since MEF2 family member MEF2A and MEF2D are transcription factors bound to *mFshβ* promoter in αT3-1 cells (Lim, Luo et al. 2007), we anticipated that ACTN4 may also regulate transcription of *mFshβ* gene through its CaM-like domain. To test this hypothesis, the full-length ACTN4 and its truncated mutant ΔCaM-ACTN4, without the CaM-like domain, were transiently transfected into LβT2 cells. The protein expression of each construct was analyzed by western blot (Figure 6.7 (a)). The effects of these proteins on the mRNA level of *mFshβ* gene were assessed by real-time PCR. The result demonstrates that ACTN4 OE promotes mRNA level of *mFshβ* gene to nearly 1.5 fold; however ΔCaM-ACTN4 has no detectable effect (Figure 6.7 (b)).

Furthermore, luciferase assay was performed to evaluate the effect of ΔCaM-ACTN4 on the *mFshβ* promoter activity (Figure 6.7 (c)). The result shows similar trends as that of real-time PCR. Compared to the control, ACTN4 significantly increased *mFshβ* promoter activity for ~2 fold, while this function was almost abolished after deleting the CaM-like domain. Our findings suggest that the C-terminal CaM-like domain of ACTN4 might be involved in the regulation of *mFshβ* gene expression.

(a)


pXJ40-FLAG
FLAG-ACTN4
FLAG-ACTN4 ΔCaM
ACTN4
FLAG
actin

(b)


Normalized mRNA levels (fold change over control)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
control
ACTN4 OE
ΔCaM-ACTN4

(c)

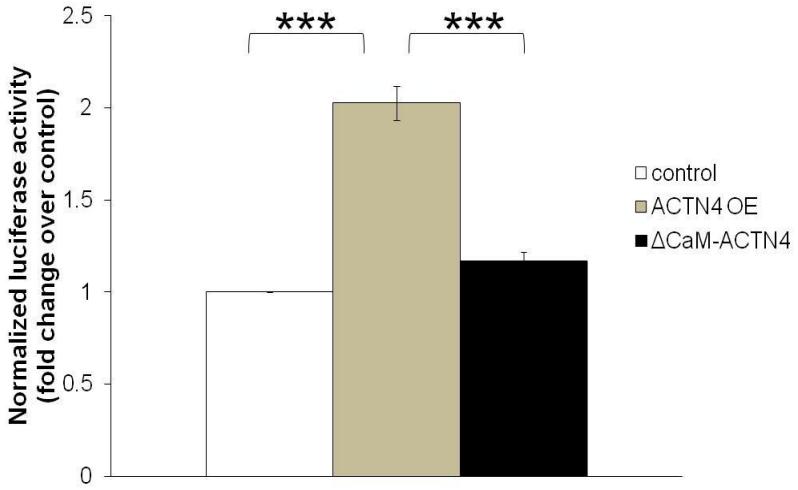


Figure 6.7 ACTN4 induced transcription activation of mFshβ requires its CaM-like domain.

(a) pXJ40-FLAG, ACTN4-FLAG and ΔCaM ACTN4-FLAG were transiently transfected into LβT2 cells. The expression of full length and truncated ACTN4 was detected by western blot with actin as loading control. (b) Real-time PCR of regulation of ACTN4 on the *Fshβ* gene. Total RNAs were extracted from LβT2 cells transfected with above plasmids. Quantitative real-time PCR was performed as described in Figure 6.5. (c) Luciferase assay of ACTN4 on *mFshβ* promoter. *mFshβ* promoter-luciferase constructs were co-transfected with either ACTN4-FLAG, ΔCaM ACTN4-FLAG or pXJ40-FLAG. Firefly luciferase values were analyzed as described in Figure 6.6. *P<0.05; ***P<0.001.

6.3 Discussion

Here we propose three possible roles of ACTN4 in GnRH-mediated transcriptional activation. Firstly, ACTN4 may promote the transcription of *mFshβ* through antagonizing HDACs from its promoter. It has been reported that the ACTN4 and MEF2 binding sites on HDAC7 are largely overlapped. Furthermore, the CaM-like domain of ACTN4 is critical for its interaction with HDAC7. Because of these, ACTN4, through its CaM-like domain, can compete with MEF2 for binding to HDAC7 thus potentiate MEF2 transcription activity (Chakraborty, Reineke et al. 2006). MEF2 is a known transcription factor bound to *Fshβ* promoter in αT3-1 cells (Lim, Luo et al. 2007), the released inhibition of MEF2 through dissociation with HDAC7 may therefore promote the transcription of *Fshβ* gene. Besides, ACTN4 may also regulate the direct inhibition of *mFshβ* promoter through releasing the HDACs from binding to the promoter. It has also been reported that GnRH facilitates the expression of *mFshβ* gene in αT3-1 cells through activating Ca^{2+}/CaMKI and phosphorylating the HDACs (Lim, Luo et al. 2007). The phosphorylated HDACs dissociate from *mFshβ* promoter, thus the HDAC-mediated repression of *mFshβ* gene is released. This finding indicated that GnRH stimulation may trigger the Ca^{2+} signaling pathway as well. Thus, It is possible that ACTN4 is involved in regulating the transcription of *mFshβ* gene through GnRH-induced activation of Ca^{2+} signaling pathway, causing HDAC phosphorylation and relief of gene repression.

Secondly, it is also possible that ACTN4 may stabilize chromatin remodeling complexes to activate *mFshβ* transcription. In the study of human cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), association of ACTN4/ NF-Y has been identified and the role of ACTN4 has been proposed in assisting NF-Y to recruit chromatin

remodeling complexes or directing ACTN4/NF-Y associated gene into active transcription (Poch, Al-Kassim et al. 2004). Given that murine *Fshβ* promoter also contains a half AP-1/NF-Y binding site (Jacobs, Coss et al. 2003), and this site is required for the maximal induction of GnRH in LβT2 cells (Coss, Jacobs et al. 2004), we speculate that ACTN4 may help to stabilize chromatin remodeling complexes or orchestrate target genes to facilitate the transcription activation induced by GnRH.

Thirdly, ACTN4 may link Ca^{2+} signaling and MAPK signaling during the GnRH-mediated signal transduction pathways. It is known that the MAPK pathway is important for GnRH signaling in the regulation of synthesis of gonadotropins. Previous study on CaM in αT3-1 cells suggested that it may act as a Ca^{2+} sensor, which induces Ca^{2+} flux and consequently leads to extracellular regulated kinase (ERK) activation within the GnRH signaling (Roberson, Bliss et al. 2005). It is evident that ERK is a crucial intermediate to transduce the signal from GnRH to *Fshβ*. Inhibition of ERK decreased GnRH-induced *mFshβ* promoter activity in LβT2 cells (Coss, Jacobs et al. 2004) and attenuated GnRH-stimulated mRNA level as well as FSH release in male rat primary pituitary cultures (Haisenleder, Cox et al. 1998). As ACTN4 harbors the CAM-like domain, it may also mediate the activation of ERK through GnRH-induced Ca^{2+} signaling. In this study, we demonstrate that ACTN4 may play an essential role on the *mFshβ* promoter through its CaM-like domain. Therefore, it is plausible that ACTN4 also provides a link between Ca^{2+} signaling and MAPK signaling during the GnRH-mediated signal transduction pathways (Naor, Benard et al. 2000). The above mentioned hypothesis was summarized in Figure 6.8. Further experiments will be required to verify this complex signaling process.

To summarize, we have demonstrated that the nuclear translocation of ACTN4 is involved in the regulation of *mFshβ* gene transcription by GnRH, and its CaM-like domain is required for this function. Our findings suggest that GnRH-induced ACTN4 nuclear translocation may facilitate the transcriptional activation of *mFshβ* gene, possibly through mediating the crosstalk of Ca^{2+} signaling with the MAPK pathways.

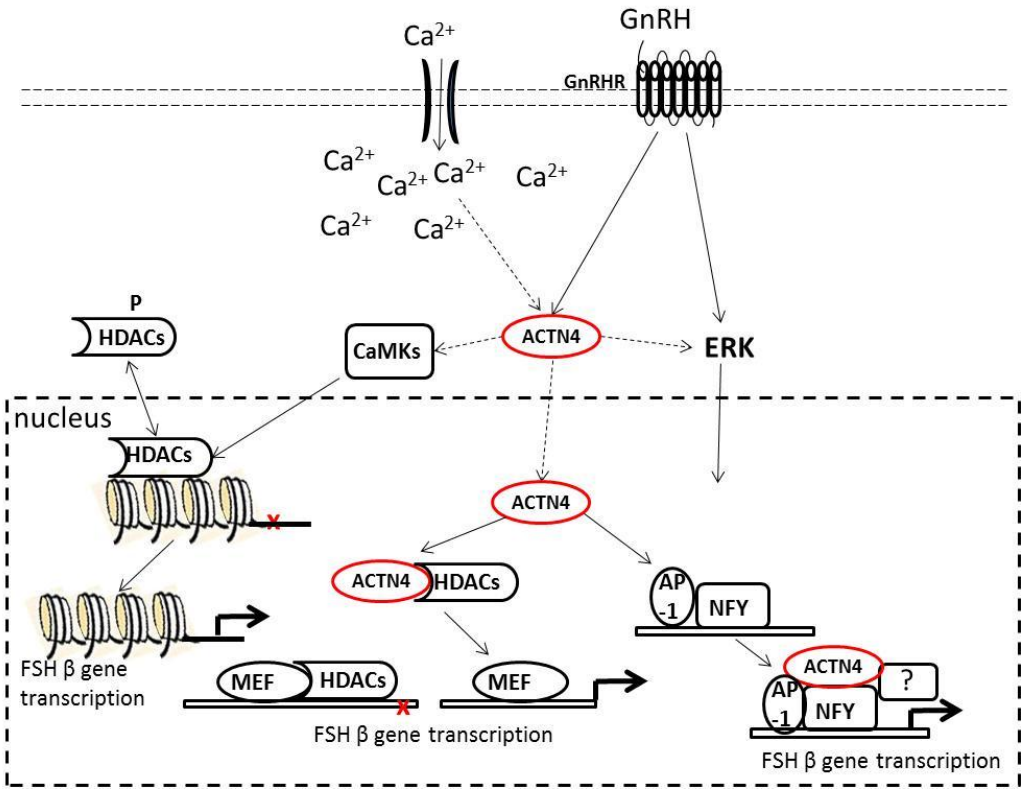


Figure 6.8 Proposed functional roles of ACTN4 in the regulation of FSHβ gene transcription.

ACTN4 may function through regulating CaMKs and consequently dephosphorylates and releases HDACs from binding on the FSHβ promoter; ACTN4 may also binding with HDACs and subsequently prevent the HDACs binding to the MEF, as a result to induce FSHβ gene transcription; ACTN4 can bind to NFY and recruit other factors to induce FSHβ gene transcription; ACTN4 may also mediate the crosstalk between Ca^{2+} signaling and MAPK pathway, possibly through ERK.

Chapter 7. Conclusion and future directions

7.1 Conclusion of the Study

GnRH is the first key hormone of reproduction. GnRH analogs are extensively used in *in vitro* fertilization, and treatment of sex hormone-dependent cancers. An understanding of the GnRH signaling mechanisms is crucial for establishing interactive signaling map, pointing to targets for new drugs. However, during the past 10 years, the majority of the study was focused on GnRH-induced MAPK cascades and the MAPK mediated transcriptional regulation of gonadotropins. Although the knowledge of these fields are quite solid and specified, in terms of new drug development, however, the knowledge is too limitated. Thus there is a need to discover new pathways and new regulatory mechanisms initiated by GnRH. As such we performed high-throughput proteomic studies in LβT2 gonadotrope cell line.

As described in chapter 4, we began with cICAT proteomic profiling, where comparison between whole-cell proteome from 4 h mock-treated and GnRH-treated LβT2 cell lines was performed allowing an identification of differentially regulated low abundant signaling proteins. We found several significantly regulated proteins were involved in PTMs, suggesting the involvement of new GnRH-regulatory mechanisms. Furthermore, we found a few potential mediators which may mediate GnRH response to Wnt/β-catenin signaling. Based on our finding we proposed a model showing the crosstalk between GnRH signaling and Wnt signaling. We proposed that β-catenin may be stabilized by two distinct strategies. One is through β-catenin phosphorylation-dependent mechanism, which may be mediated by either GSK-3β Ser 9 dependent-mechanism, or GSK-3β Ser 9-independent mechanism or both. The other is through β-catenin phosphorylation-

independent mechanism, which is mediated by Cacybp-containing E3 ubiquitin complex, and this pathway is possible to connect Wnt signaling with MAPK cascades.

Although our cICAT proteomic profiling study allows the identification of several signaling molecules, the overall number of the proteins identified by ICAT is relatively small thus not sufficient to interpret the GnRH signaling from a comprehensive perspective. So in Chapter 5 we used an alternative proteomic method, iTRAQ-based proteomics experiment, to perform the nuclear proteomic comparative profiling in mock-treated and GnRH-treated LβT2 cells. Rather than just a repetitive study, our iTRAQ study changed the GnRH treatment from continuous treatment to pulse treatment, which better represent the physiological condition (Lawson, Tsutsumi et al. 2007). In iTRAQ study, we identified a number of differentially regulated proteins with novel functional roles such as mRNA processing, translation, and chromosome organization, suggesting multifunctional roles of GnRH in the regulation of gonadotropins. Furthermore, we identified several potential Ca^{2+} signaling regulators, which are involved in regulating calcium concentration, CaM-binding and CaM downstream signaling, were differentially regulated by GnRH pulse frequency. These findings provide more evidence suggesting the crucial role of Ca^{2+} signaling in decoding the GnRH pulse frequency.

Finally we validated one of the iTRAQ identified proteins, ACTN4, which was found to be involved in Ca^{2+} signaling. We proved that ACTN4 is a positive regulator of FSHβ gene transcription, and the CaM-like domain is required for this function. The results also suggested that ACTN4-induced transcriptional activation of *mFshβ* gene, possibly through mediating the crosstalk of Ca^{2+} signaling with the MAPK pathways.

In summary, our study is the first comprehensive proteomic study which was performed in gonadotrope cell line to explore the GnRH signaling. Our finding identified novel functional roles of GnRH and novel pathways mediating GnRH response to gonadotropins (Figure 7.1). Our findings may provide valuable clues and suggestions for future researches to continue exploring the interactive GnRH pathway networking, as well as identifying potential drug targets for the therapy of reproductive aberrance and deficiency related diseases.

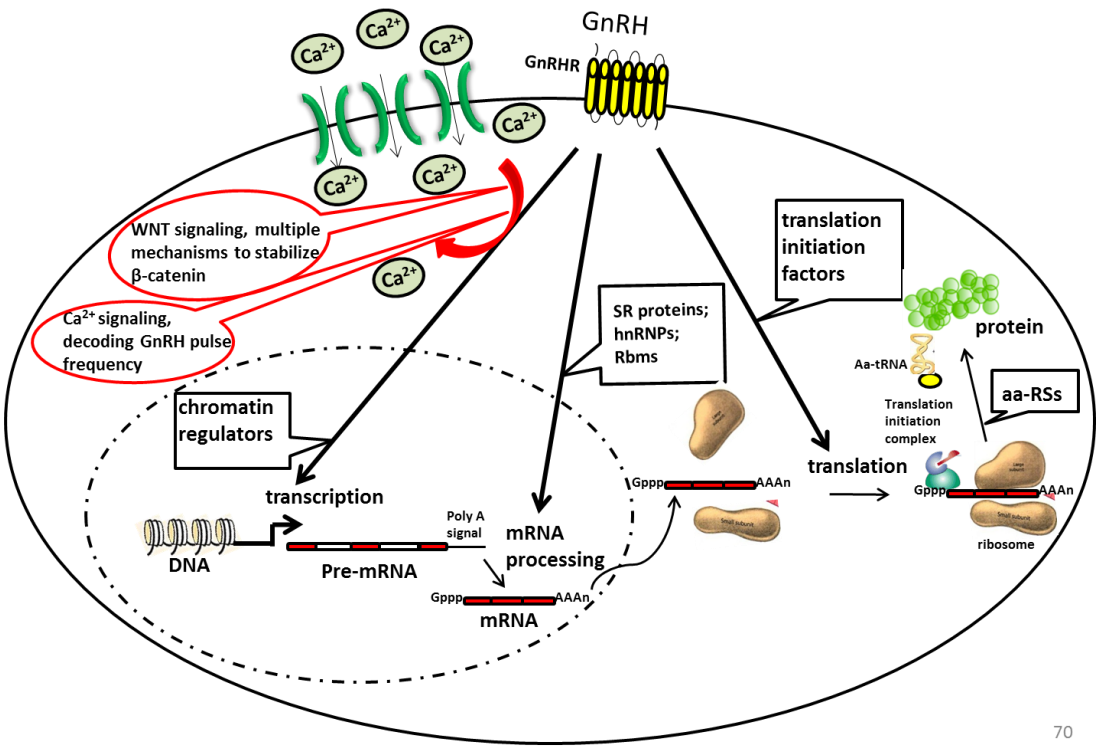


Figure 7.1 Summary of the identification of novel functional roles of GnRH and novel pathways mediating GnRH stimulation of gonadotropin production.

In this proteomic study, we identified a number of differentially regulated proteins with novel functional roles such as mRNA processing, translation, and chromosome organization, suggesting multifunctional roles of GnRH in the regulation of gonadotropins. Moreover, the novel signaling pathways mediating transcription of gonadotropins, include Wnt and Ca2+ signaling, have been revealed. For Wnt signaling, the mechanisms of β-catenin phosphorylation-dependent and phosphorylation-independent pathways were proposed (Figure 4.9). For Ca2+ signaling, a number of CAM binding proteins were revealed and the downstreams CAMKs pathway and calcineurin pathway were discussed (
Figure 5.11).

7.2 Future directions

Based on the new findings obtained toward GnRH signaling and gonadotropins regulation, we suggest focusing the future works on the following areas:

- Wnt signaling

We have identified several candidates which may mediate GnRH response through Wnt signaling, further work will be done to verify their functions based on the model proposed in Fig. 4.9. The mechanism of β-catenin stabilization can be investigated as shown in Figure 7.2.

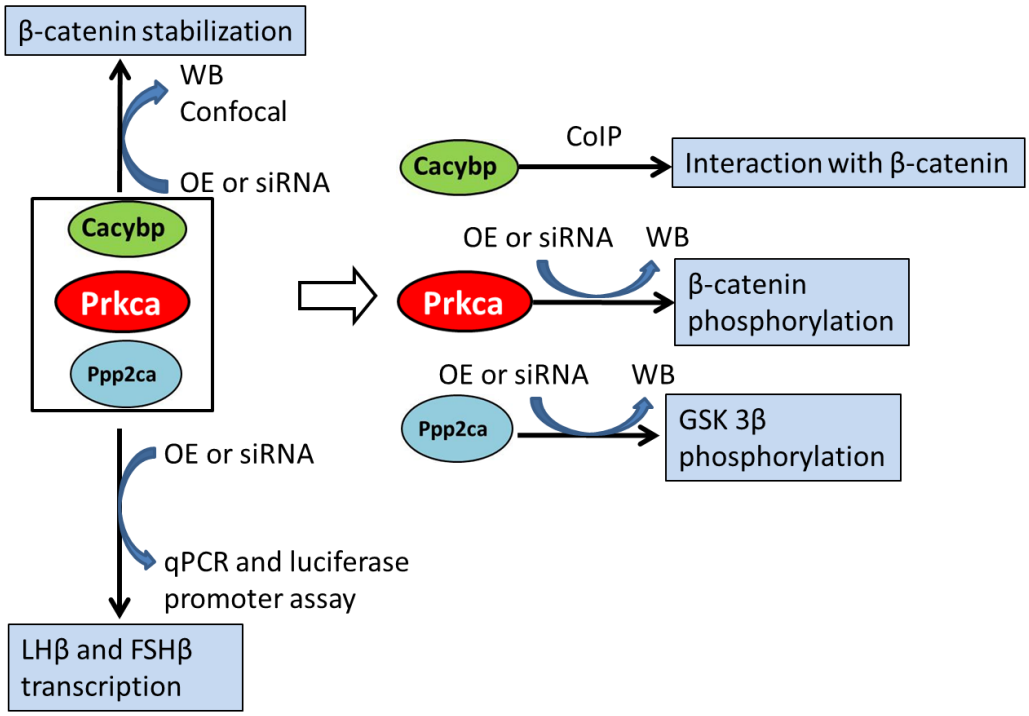


Figure 7.2 Investigation of GnRH-induced β-catenin stabilization mechanism.

- **Ca^{2+} signaling**

We have identified crucial factors induced by GnRH, which are involved in regulating calcium concentration, CaM-binding and CaM downstream signaling. Further work will be done to verify their functions based on the model proposed in Fig 5.10. The involvement of the candidates in Ca^{2+} signaling can be investigated as shown in Figure 7.3.

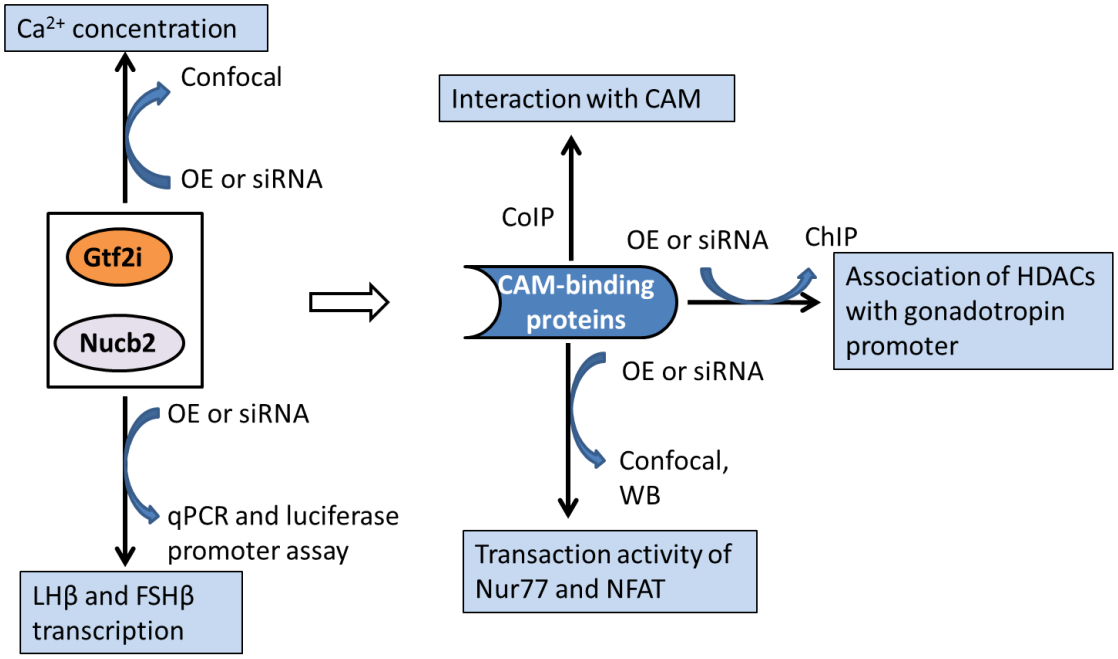


Figure 7.3 Investigation of the involvement of Ca^{2+} concentration regulators, CAM-binding proteins in the regulation of gonadotropins.

- **Chromosome remodeling**

We identified several chromatin regulators. They are possibly involved in regulating two chromosome co-repressor complexes, which recruit HDACs to the chromosome and inhibit the transcription (Figure 5.10). Further work will be performed to validate the involvement of these factors (Figure 7.4)

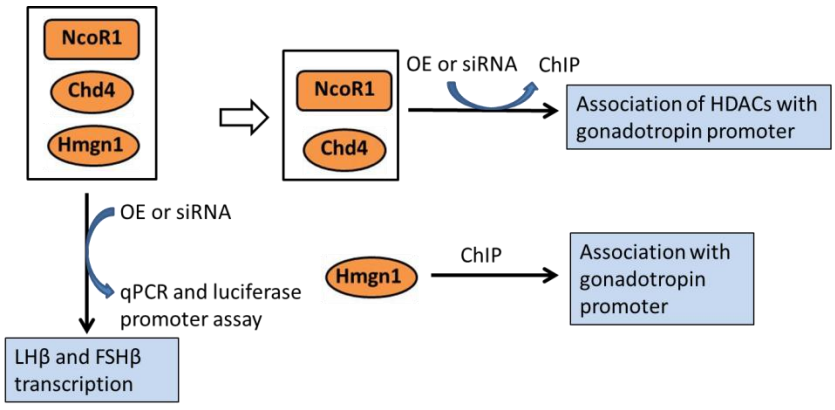


Figure 7.4 Investigation of the proteins involved in chromosome organization.

- **Promoter pull-down based proteomic study**

Several transcription factors have putative binding site (s) on LHβ and FSHβ promoters (Halvorson, Ito et al. 1998; Dorn, Ou et al. 1999; Jacobs, Coss et al. 2003). As these transcription factors are able to recruit other coactivators to the corresponding promoters, we designed a promoter pull-down based proteomic study (Figure 7.5). We expect that coactivators for each specific promoter can be identified using this method. Compared to our current proteomic profiling, this new approach may provide more specific findings of how GnRH response is mediated to each gonadotropin promoter. We did a preliminary test to check the applicability of the promoter pull-down experiment by using LHβ intact and mutant promoters. As shown in Figure 7.6, this method is efficient and specific thus can be used for further study.

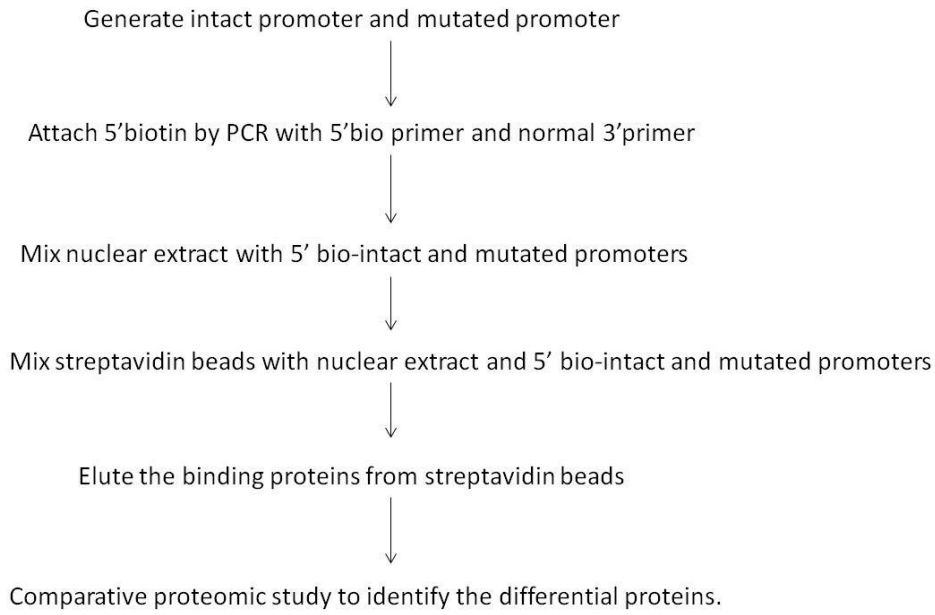


Figure 7.5 Strategy and flowchart of promoter pull-down based proteomic study.

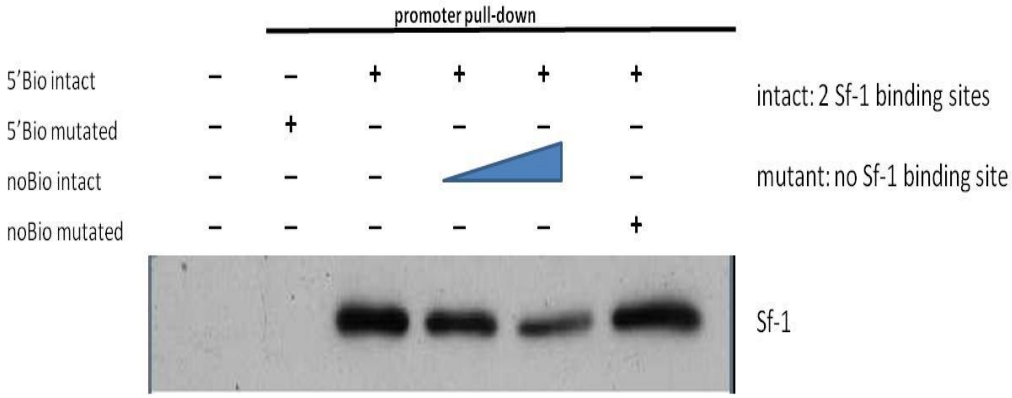


Figure 7.6 Sf-1 promoter pull-down experiment.

5'bio-mutant promoter without Sf-1 binding site fails to pull down Sf-1 (Lane 2), while 5'bio-intact promoter with two Sf-1 binding sites pulls down Sf-1 successfully (Lane 3). Combining the 5'bio-intact promoter with the competitor, nobio-intact promoter shows thatincreasing amount of competitor results in reduced amount of Sf-1 pulled down (Lane 4, 5). The nobio-mutant promoter can't compete with 5'bio-intact promoter, so there is no difference of Sf-1 pull down between Lane 6 and Lane 2. The input Sf-1 is nearly undetectable (Lane1), while the pull-down of Sf-1 is obvious (Lane 3 and 6), indicating high efficiency and specificity of this experiment.

- **Pulse frequency operation model**

In our study we tried to use 30 min and 4 h GnRH treatments to mimic the high and low GnRH frequencies. We did observe several proteins were differentially regulated by 30 min and 4 h GnRH-treatments, which may help to explain the previous observation that GnRH pulse frequency differentially regulate the expression of LHβ and FSHβ genes (Bedecarrats and Kaiser 2003). However, compared to the continuous GnRH-pulsatile secretion in the physiological condition, our experiment can only mimic single pulse. Lawson's group has developed a pulse generating system, named LβT2 perifused system, which has only been applied to the genomic study (Bedecarrats and Kaiser 2003; Lawson, Tsutsumi et al. 2007). Thus we may borrow their idea and apply the pulse generator to our future proteomic study.

- **From *in vitro* study to *in vivo* study**

We have mentioned in Chapter 1 that there were some conflicting results towards GnRH regulation of gonadotropins. These conflicting results were suggested due to different cell type, promoters, species, and cell culture conditions (Naor 2009). Although work published in the past few years have clarified some of these ambiguities, more work is required to elucidate the molecules that mediate the hormonal activation of gonadotropins in different species and under various physiological settings and not just those that can bind and activate transcription *in vitro*. This will inevitably require greater use of animal models.

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